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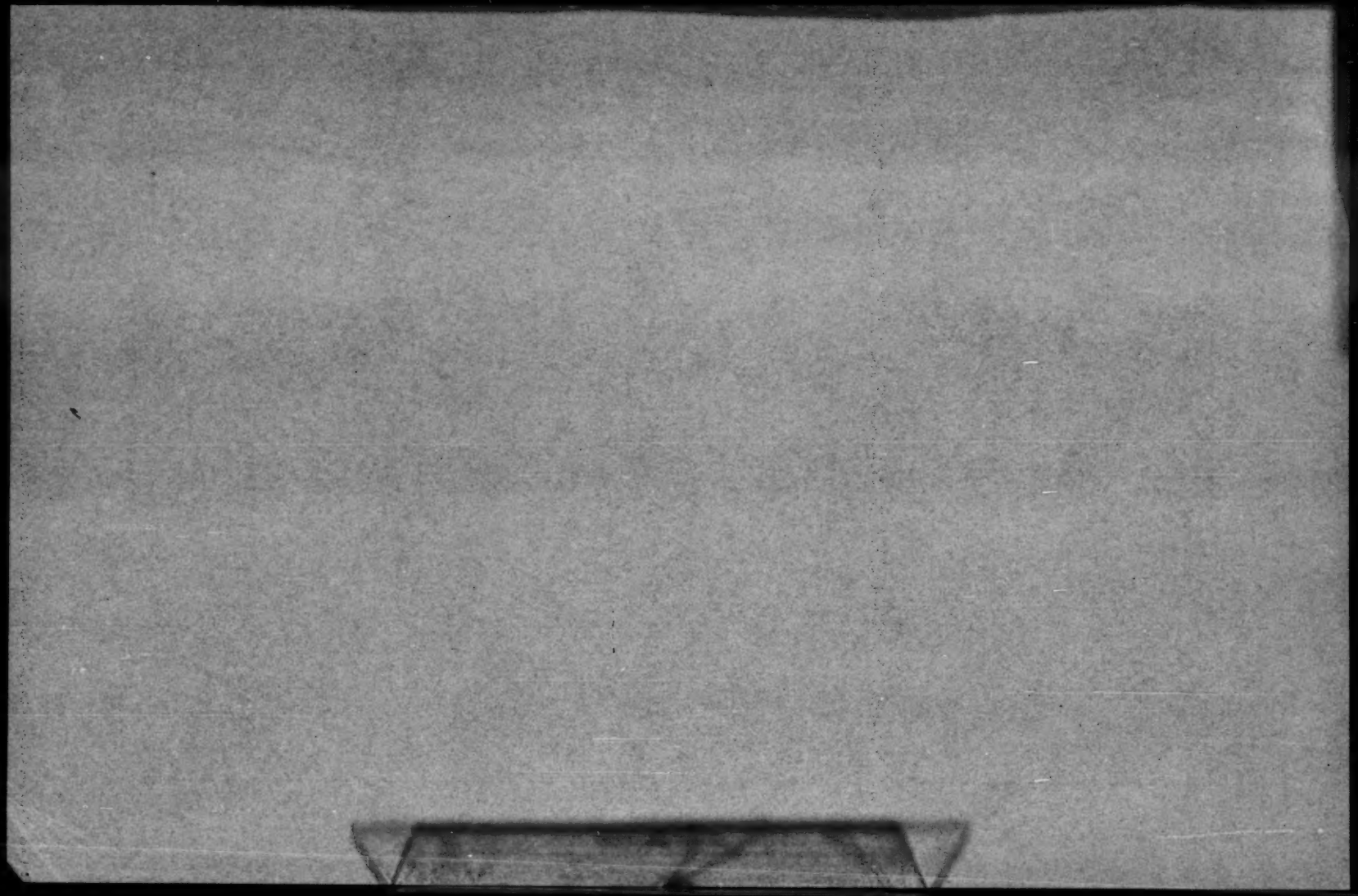

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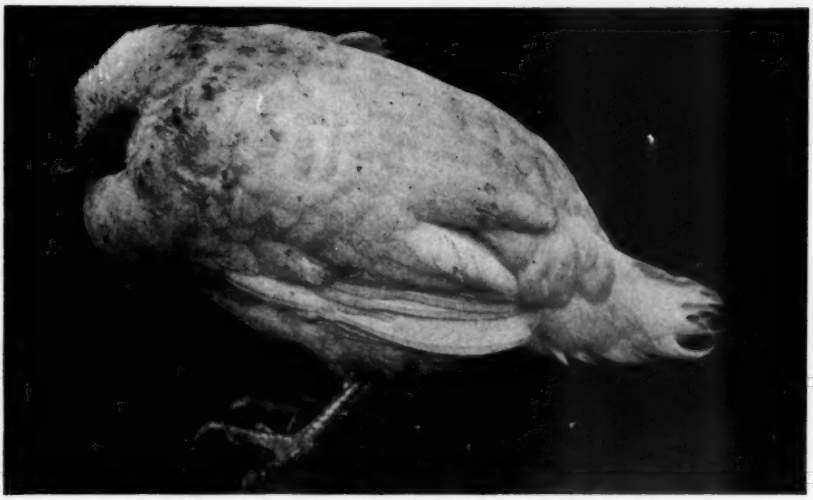

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Response of Cattle to Brucella Following Sexual Stimulation and Brucella Exposure During Early Calfhood

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Lafayette, Indiana

IT IS DEFINITELY known that sexually immature cattle resist infection by *Brucella abortus*. Also, castrated cattle show little or no susceptibility to Brucella. Hence, it has been stated many times by many competent investigators that brucellosis infection in cattle has a definite predilection for functioning reproductive tissues. This lack of susceptibility to infection by young calves had led to the adoption of successful plans of brucellosis control based on the isolation of young calves from infected herds, maintenance of these animals on a separate herd basis after the termination of first pregnancy, and replacement of parent stock with such offspring reared in a Brucella-free environment. The desirability of investigating the mechanism behind this natural resistance could hardly be over estimated. If there should be a usable relationship between sex hormone level and susceptibility to Brucella infection, it would be desirable to investigate the possibility of altering sex hormone levels in the young calf in an attempt to stimulate the immunity-producing mechanisms of the tissues at an early age. Such conceivably might make possible vaccination with fully virulent Brucella at an age younger than 4 months, with the hope of increasing resistance to infection by *Br. abortus*. Such practice might also increase the effectiveness of calf vaccination by limiting variations naturally resulting from the wide

TABLE I—Blood Serum Agglutination Response in Calves Following Stimulation with Female Sex Hormones

Animal No.	Pre-exposure titer	Agglutination response: 9	30	Days after subcutaneous exposure to *Brucella abortus*: 60	90	174	401	456
				Gonadotropin				
1	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	P – – – –	– – – – –	PT – – –
2	– – – – –	+ + + + P	+ + + + +	+ + + T –	+ + – – –	– – – – –	– – – – –	– – – – –
3	– – – – –	+ + + P –	+ + + + +	+ + + P T	+ P – – –	T – – – –	– – – – –	– – – – –
4	– – – – –	– – – – –	+ + P T –	P T – – –	– – – – –	– – – – –	T – – – –	– – – – –
				Estrogen				
5	– – – – –	+ + + P –	+ + + P T	P T – – –	P T – – –	T – – – –	– – – – –	– – – – –
6	– – – – –	+ + + + T	+ + + + +	+ + P T –	+ + P – –	P T T – –	P T – – –	– – – – –
7	– – – – –	+ + + + P	+ + + P T	+ + + P T	+ + – – –	P T – – –	+ + – – –	P T – – –
8	– – – – –	+ + P T –	+ + + P –	+ + T – –	+ P – – –	– – – – –	P T – – –	P – – – –
				Controls				
9	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –
10	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –
11	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –
12	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	– – – – –	+ P T – –

+ = Complete agglutination. P = Partial agglutination. T = Trace of agglutination. – = Negative agglutination.

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The authors are indebted to Dr. L. M. Hutchings, Dr. F. N. Andrews, and Miss Doris E. Bunnell for their part in carrying out this work.

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range in age at which the first estrus occurs.

Past investigations indicate that blood serum agglutinins in young calves arise not as a result of activity of the natural agglutinin-producing process of the calf itself but from the ingestion of colostrum or milk containing agglutinins from Brucella. Huddleson[1, 2] found that agglutinating and complement-fixing bodies for *Br. abortus* are rarely demonstrated in the blood of calves as a result of ingesting naturally infected milk. He later demonstrated that calves ingesting milk containing *Br. abortus* from cows infected either naturally or artificially with *Br. abortus* failed to react in a manner which would indicate the establishment of the organism in the body tissues. These results correspond to those obtained by Quinlan[3] who found no evidence of infections persisting in 39 calves showing a blood reaction at birth or following the ingestion of colostrum from cows infected with *Br. abortus*. Carpenter[4] was successful in isolating *Bacterium abortus* from the lymph nodes centripetal to the oral cavity and pharynx as well as from the mesenteric lymph nodes. He found that the organism remained in the lymph nodes of the calf, especially the submaxillary, atlantal, and suprapharyngeal for at least four weeks after the animal was removed from an infected environment and fed on milk not containing the *Bact. abortus* organism. According to his results, the age of the calf had little or no effect on invasion of the tissues by the organism. Interestingly, blood serum agglutination reactions in such young animals were usually negative.

In our work, calves of about 1 month of age were injected with gonad-stimulating and estrogenic hormones in an attempt to simulate the pubertal state. Later immunological studies were undertaken to determine if the resistance of pregnant heifers had been altered as a result of hormone administration combined with exposure to virulent *Br. abortus* during early calfhood.

EXPERIMENTAL

Twelve young female calves were used in the study. The history of each animal was unknown except for verbal assurance from each owner that the animal purchased was under 1 month of age. The calves were tested for the presence of Brucella agglutinins using the rapid plate test as described by Huddleson,[5] employing plate antigen prepared by the United States Bureau of Animal Industry at serum dilutions 1:25, 1:50, 1:100, 1:200, and 1:400. No animal reacted to the plate test at the time of purchase.

The calves were divided into three equal groups and treated as follows:

Group 1 was injected subcutaneously with 10 units of gonadogen (pregnant mare's serum, Upjohn) on alternate days for a series of seven injections. This was followed by 1,250 international units of chorionic gonadotropin (human pregnancy urine, Upjohn) on alternate days for a series of six injections, after which gonadogen injections were resumed for a series of seven injections using 15 units per injection.

Group 2 was treated in a similar manner but 2.5 mg. doses of estrogenic substance (diovocylin, Ciba) were substituted for the gonad-stimulating hormones.

TABLE 2—Blood Serum Agglutination Response and Cultural Results in Heifers at Termination of Pregnancy

Animal No.	Titer before reëxposure	Agglutination response: Days after reëxposure					Parturition	Cultural results at parturition
		18	31	74	108	144		
				Gonadotropin				
1	P T – – –	P T – – –	P T – – –	+ P T T T	P T – – –	++P T –	A	pos.
2	– – – – –	T T – – –	T T – – –	P T T T –	– – – – –	– – – – –	N	neg.
3	– – – – –	– – – – –	T – – – –	– – – – –	– – – – –	– – – – –	N	neg.
4	– – – – –	++P T T	+ P T T –	+ P T T T	T – – – –	P – – – –	N	neg.
				Estrogen				
5	– – – – –	T – – – –	T – – – –	P T T – –	P – – – –	– – – – –	NP	
6	P T – – –	– – – – –	T – – – –	P T T T –	T – – – –	P – – – –	N	neg.
7	P – – – –	– – – – –	T T – – –	T – – – –	++P T T	++++P	A	pos.
8	– – – – –	T – – – –	– – – – –	T T T – –	– – – – –	P – – – –	A	pos.
				Controls				
9	– – – – –	– – – – –	– – – – –	+ P T – –	+++++	++++P	A	pos.
10	– – – – –	P T – – –	T – – – –	– – – – –	– – – – –	– – – – –	N	neg.
11	– – – – –	– – – – –	– – – – –	T – – – –	P T – – –	– – – – –	A	pos.
12	+ PT – –	T T – – –	PPTT– –	T T – – –	+++++	+++– –	A	Membranes and fetus unavailable

+ = Complete agglutination. P = Partial agglutination. T = Trace of agglutination. – = Negative agglutination. N = Normal. A = Aborted dead calf. Positive indicates recovery of *Brucella abortus* from colostrum and/or fetal placenta and calf. NP = not pregnant.

Group 3 served as the untreated control unit.

All calves were exposed to a laboratory culture of *Br. abortus* three times during the period of hormone administration. The first exposure was per conjunctiva two weeks after starting hormone injections using about 200 million organisms, the second exposure similarly one week later using about 64 million organisms, followed in two weeks by a third subcutaneous exposure of about 800 million organisms. The three groups were kept in a common enclosure and managed as a unit. Blood samples were collected at weekly intervals to determine blood serum agglutination response. The standard rapid plate test was used as described above.

At approximately 1 year of age, the heifers were bred. One of the animals in group 2 failed to conceive after repeated breedings. After pregnancy had been established in the others as determined by rectal palpation, all animals were exposed to approximately one million *Br. abortus* organisms by the conjunctival route. The suspension of Brucella used was injected into guinea pigs at the time of exposure to determine its pathogenicity for this species. The exposure strain was recovered from an aborted calf and conformed to the standard characteristics of *Br. abortus* as determined by sulfur gas production, CO_2 requirement, and dye plate growth inhibition. At the termination of pregnancy, fetal tissues, placentas, and colostrum were obtained when possible and examined for the presence of Brucella by direct cultural and guinea pig inoculation procedures.

RESULTS

Both hormonally stimulated groups of calves exhibited increased sexual activity as measured by estrous symptoms and udder development. Blood titers indicative of infection were elicited ten days following subcutaneous exposure in groups 1 and 2 (table 1). Agglutination titers persisted at a rather high level for approximately two months, after which they began to recede until they were essentially negative after six months.

Guinea pigs inoculated with a suspension of exposure material at the time of challenge exposure developed agglutination titers positive at a serum dilution of 1:400 (highest dilution employed) and showed lesions of brucellosis at autopsy. *Brucella abortus* was recovered from the guinea pigs at autopsy. Thus, the culture used for challenge exposure was pathogenic for guinea pigs at the time of such exposure.

Variable blood serum agglutination responses were elicited following challenge exposure (table 2). One animal in group 1, 2 animals in group 2, and 3 animals in group 3 aborted. *Brucella abortus* was recovered from the colostrum and fetal membranes and/or placentas in all recorded abortions except 1 of the controls that had an unobserved abortion while on pasture. The strains of *Br. abortus* recovered from heifers 1, 8, and 11 did not require CO_2 for primary isolation but did conform to the other characteristics of *Br. abortus*.

DISCUSSION

The results of this and other experimental work being carried on at this station indicate that susceptibility to Brucella infection may be associated with functional reproductive tissues. All animals in the experimental group showed gross udder enlargement. Indeed, several in each group produced milk and 1 individual (calf 6) developed a mastitis of the left rear quarter.

It is interesting to note that the experimental animals developed active infection as evidenced by persistent agglutination titers while the controls failed to develop agglutinins for Brucella throughout the prechallenge period.

Aside from a possible alteration in abortion rate, therefore, the results of this trial demonstrate that the early exposure of calves to *Br. abortus* under unnatural physiological conditions had little effect on susceptibility to brucellosis during the first pregnancy.

SUMMARY

1) Young female calves injected with gonad-stimulating hormones and exposed to *Brucella abortus* before puberty became infected with Brucella as measured by blood serum agglutination response. Control calves (without hormone stimulation) failed to become infected when exposed in a similar manner.

2) The agglutinins produced in the experimental calves persisted at rather high levels for two months after which serum agglutination titers gradually receded until they were essentially negative after six months.

3) Challenge exposure to virulent *Br. abortus* of these animals during their first pregnancy failed to show increased resistance to *Br. abortus* infection as a result of prepubertal infection with brucellosis,

although there was a possible alteration in the abortion rate observed.

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Comparative Tests of the Protective Value of Huddleson's Mucoid and Strain 19 Vaccines in Cattle. II

B. H. EDGINGTON, D.V.M.; N. B. KING, B.S., D.V.M., M.S.;
NORMA FRANK, B.S., M.S.

Wooster, Ohio

NUMEROUS ATTEMPTS to develop means for the control of bovine brucellosis have been reported. It would serve no worthwhile purpose to review at this time the early efforts aimed at the development of a protective vaccine against the disease, other than to state that no outstanding success in the use of agents incapable of producing infection of the vaccinated animals was attained prior to the development of Brucella strain 19 vaccine by the United States Bureau of Animal Industry (BAI).

The consensus resulting from several years' experience in the use of strain 19 vaccine is that it produces a very serviceable resistance but not an absolute immunity to *Brucella abortus* infection in cattle. Results obtained with other bacterial vaccines give slight encouragement to a hope that Brucella vaccines will afford absolute protection under all conditions of challenge.

Reactor agglutinin titers following administration of strain 19 vaccine, particularly in adult vaccination, may persist for several months. This is unfortunate, since such titers can not be distinguished from those that result following virulent infection. This condition is frequently a disturbing factor to disease control efforts wherein identification of infected cattle is based upon the results of the agglutination test.

Obviously, any vaccine not causing this agglutinin development but still affording a satisfactory plane of resistance without danger of establishing infection in the vaccinated animal would represent a valuable aid to the problem of brucellosis control.

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The authors desire to express their grateful appreciation of the assistance rendered by Mr. Homer Gall, Ohio Department of Public Welfare, in selection of the experimental animals and to the Central Ohio Breeding Association for its generosity in supplying the semen used for insemination of the heifers.

Huddleson in 1947[1] reported the results of investigations in the immunization of guinea pigs with a mucoid phase growth of Brucella. He observed that live cell suspensions of certain mucoid phase growths of *Brucella suis* and *Brucella melitensis* injected into normal guinea pigs resulted in a high degree of immunity against experimental infection with all three species of Brucella.

Expanding his investigations to include cattle, Huddleson in 1948[2] reported results obtained in 24 herds having a total of 1,127 animals that were given a vaccine prepared from a mucoid phase growth of *Br. suis*. Brucella infection was of recent development in the majority of these herds. Nonvaccinated animals were not available for use as controls in these herds. The presence or absence of brucellosis in the animals was based principally upon the serum agglutinin titers obtained in tests made at, or shortly before, the time of vaccination and at intervals over an observation period of twelve to fourteen months.

The initial agglutination tests of the animals showed 772 as negative, 158 as suspicious, and 197 as positive reactors. Forty of the 772 initially negative animals were removed from the group during the experiment. Twenty-five of the remaining 732 animals developed doubtful and 23 reactor agglutinin titers.

Referring to the work of McEwen *et al.*[3] and Bendixen and Bloom,[4] Huddleson calls attention to the period of more than 200 days that may elapse before cattle show a significant agglutinin titer after a known exposure to *Br. abortus* and states, "It was, therefore, not surprising to find 23 negative animals in a few of the herds showing evidence of infection after the injection of the vaccine."

Discussing titer development and recession that followed administration of the mucoid vaccine, Huddleson observed that, following subcutaneous injection of 10^{10} viable cells of the mucoid phase growth into noninfected adult cattle or calves, the blood serum agglutinin titers do not develop higher than 1 : 100 and as a rule a titer of 1 : 25 is not present ninety days after the injection.

The over-all results of this investigation appeared to offer encouragement as to the protective value of the vaccine used.

These investigations engaged the attention of workers in this field of research and an enthusiastic demand by many herd owners for

use of the mucoid vaccine in their animals. Experimental use in Ohio of Huddleson's Brucella M vaccine (hereinafter designated as M) was authorized by Dr. H. G. Geyer, state veterinarian, under permission to obtain the vaccine granted by Dr. B. T. Simms, chief, United States Bureau of Animal Industry. Edgington and King[5] in 1949 gave a progress report on results obtained in a comparative test of M and strain 19 vaccines used in adult beef cattle which were later challenged by experimental exposure with a virulent strain (2308) of *Br. abortus*. As a result of this exposure in the group of 7 nonvaccinated controls, 3 aborted and 1 had a premature birth. *Brucella abortus* was recovered from either the aborted fetus or milk of the dam in each of the abnormal births. Each of the 8 M and 11 strain 19 vaccinated cows had normal calvings. *Brucella abortus* was recovered from only 1 of the vaccinated cows. This cow had received M vaccine.

Results presented in the present report were obtained in the second series of experiments designed to furnish information on the protective value of Huddleson's Brucella M vaccine.

PROCEDURES

Blood Serum Agglutination Tests.—The usual tube method of testing was used throughout the experiment for titer determinations. Serum in twofold dilutions ranging from 1 : 25 to 1 : 12,800 and a standardized BAI antigen were used. The tests were read following incubation at 37 C. for forty-eight hours.

The Vaccines.—The M vaccine was supplied through the courtesy of Dr. I. Forest Huddleson from current production lots of the Brucella Laboratory, East Lansing, Mich. The strain 19 vaccine was obtained from the United States Bureau of Animal Industry. The vaccines were injected subcutaneously at the upper portion of the area immediately posterior to the scapula. The individual dose of vaccine injected was 1 ml. for M and 5 ml. for strain 19.

Immunity challenge.—Resistance of vaccinated and control animals was challenged by conjunctival sac instillation of virulent *Br. abortus* (BAI 2308) organisms. The concentration of viable cells used for the infecting doses was obtained by suitable dilution of a basic suspension of the culture standardized to contain 1 billion viable cells per milliliter. The final cell concentration was so adjusted that 0.1 ml. contained the entire infecting dose of 750,000 organisms for each animal.

Attempted recovery of *Br. abortus* following immunity challenge was limited to the inoculation of Tryptose-agar plates with milk, stomach content of the aborted fetus, and vaginal swabs made from the recently calving dam.

Duplicate plates were inoculated with each material. One of each sets of plates was incubated under increased CO_2. Following a minimum of four days of incubation at 37 C., colonies showing Brucella characteristics were selected for further identification. Saline suspensions of the selected cultures were used as the antigen in agglutination tests with known positive and negative *Br. abortus* serums.

Huddleson's dye plate method[6] was used for species determinations.

THE EXPERIMENT

Twenty-one Holstein-Friesian heifers obtained from a brucellosis-free herd were used in this test.

During the eighteen months between the time of their assembly and that of experimental exposure, they were maintained in pasture or winter quarters as a single herd under conditions intended to avoid opportunity for unintentional Brucella exposure.

They were considered as three groups of 7 head each for the purpose of the experiment. Group A received M vaccine, group B was given BAI strain 19 vaccine, and group C was retained as nonvaccinated controls.

The heifers showed negative agglutinin titers at 1 : 25 dilution in the initial test at the time of their assembly and on two subsequent tests at monthly intervals prior to vaccine administration.

The age at time of vaccine administration of the heifers that were ultimately exposed is shown in table 2. Originally, it was intended to use heifers between 6 and 8 months of age at the time of vaccination; however, the animals were not available for the test until they ranged in age between 8 to 15 months. The average vaccination age of the heifers in groups A, B, and C that were ultimately exposed to *Br. abortus* was 10.1; 9.6; and 11.3 months, respectively.

Breeding of the heifers was started approximately ten months following vaccine administration and was limited to a period of eighty days in an effort to obtain gestations of somewhat uniform duration at the subsequent time of Brucella challenge. The heifers were bred principally by artificial insemination which resulted in established pregnancy in 5 of the strain 19 vaccinated animals and 6 each of the M vaccinated animals and nonvaccinated controls.

Just prior to Brucella challenge, the heifers were placed in individual pens where they remained throughout the remainder of the experiment. The pens were arranged on either side of a central feedway. The pens and feedway were of concrete construction and each pen was separated from the adjoining pens by concrete partitions. The front of the pen had an entry gate adjacent to which was a removable feed bunker extending into the feedway. At the rear of this bunker was a catching stanchion used for restraint of the animal for obtaining blood samples without necessity of entering the pen.

With this arrangement it became unnecessary for the attendants to enter the pens except for daily removing of manure. The control group

TABLE I—Results of Agglutination Tests of Heifers

Group and No.	At Time of vaccination	Months After Vaccination					After *Brucella abortus* Exposure Oct. 6			At calving	Approx. 1 month after calving	Approx. 2 months after calving	Outcome gestation and date	*Br. ab.* recovered
		one	two	six	Eight	Sixteen and at exposure	Oct. 27	Nov. 19	Dec. 20					
M-Vac.														
No. 5	All animals negative in 1:25 dilution	*I-100	I-25	N-25	N-25	I-25	C-50	I-50	I-25	I-50	I-50	I-50	**N-Jan. 12	neg.
11		I-50	N-25	N-25	N-25	N-25	N-25	I-50	I-1600	I-1600	I-6400	C-12,800	P-Dec. 23	pos.
14		I-100	N-25	I-25	N-25	I-25	I-25	I-50	I-25	I-25	I-25	.....	N-Mar. 19	neg.
15		I-100	I-25	I-25	N-25	I-25	I-25	I-25	I-25	N-25	N-25	N-25	N-Feb. 20	neg.
16		I-50	N-25	I-25	N-25	N-25	I-25	C-100	I-800	I-3200	I-3200	C-1600	A-Jan. 4	pos.
20		I-50	I-25	I-25	N-25	I-25	I-200	I-200	C-400	C-400	I-6400	I-12,800	A-Dec. 24	pos.
19-Vac.														
No. 6		I-12,800	I-3200	I-1600	I-200	I-50	I-100	I-50	I-25	I-100	C-50	C-50	N-Dec. 30	neg.
7		C-12,800	I-3200	I-1600	I-400	I-100	I-400	I-200	I-200	I-100	C-200	C-100	N-Feb. 23	neg.
17		I-12,800	I-1600	I-400	I-100	I-100	I-200	I-100	C-50	I-200	C-800	I-3200	N-Feb. 11	pos.
19		I-6400	I-400	I-100	I-100	I-200	I-200	I-100	C-50	I-200	I--100	I-100	N-Jan. 4	neg.
21		I-1600	C-400	I-25	N-25	N-25	N-25	N-25	N-25	N-25	N-25	N-25	N-Dec. 30	neg.
No Vac.														
No. 1		I-25	N-25	N-25	N-25	N-25	I-100	I-3200	C-12,800	I-6400	C-12,800	C-12,800	A-Nov. 24	pos.
2		N-25	N-25	N-25	N-25	N-25	I-25	I-200	I-800	I-800	I-3200	I-6400	A-Dec. 17	pos.
3		N-25	N-25	N-25	N-25	N-25	C-100	I-800	I-1600	I-1600	I-12,800	I-6400	A-Dec. 22	pos.
4		N-25	N-25	N-25	N-25	N-25	I-25	I-100	C-800	I-400	C-6400	I-6400	P-Dec. 11	pos.
8		I-25	I-25	N-25	I-25	N-25	I-100	I-200	I-3200	I-800	I-12,800	C-12,800	A-Dec. 15	pos.
18		I-25	N-25	N-25	N-25	N-25	N-25	I-100	N-25	I-25	I-25	.....	N-Mar. 15	neg.

NOTE: Monthly tests made at intervals between those recorded showed no significant titer change and are omitted from the table for the sake of brevity.

*N = negative, I = incomplete, C = complete. Serum dilution indicated by numerals. **N = normal, calved not less than 271st day of gestation. P = premature, calf alive. A = aborted, calf dead.

of heifers was retained at one end of the series of pens. No other effort at segregation of the groups was attempted and the same attendants cared for the three groups of heifers indiscriminately. Brucella challenge of the 17 pregnant heifers was made approximately sixteen months following vaccine administration. *Brucella abortus* used for challenge was a lyophilized sample of culture 2308, lot 373, furnished by the United States Bureau of Animal Industry. The lyophilized sample was reconstituted in 0.3 ml. sterile 0.85 per cent saline solution. Tryptose-agar slants were inoculated with 0.1-ml. amounts of this suspension and incubated at 37 C. for sixty-eight hours. The resulting growth was removed with saline solution and standardized to contain 750,000 organisms per exposure dose of 0.1 ml. This volume of culture suspension was administered to each heifer, one half of the dose being instilled into the conjunctival sac of each eye.

Agglutinin titers of the heifers obtained in tests made at intervals throughout the experiment are given in table 1. All heifers showed negative titers at 1 : 25 dilution in each of the tests made prior to the time of vaccine administration. The M vaccinated animals showed but slight titer increase following vaccine administration. These titers receded to negative or incomplete at 1 : 25 in tests made two months postvaccination and remained within this limit up to the time of Brucella exposure. The strain 19 vaccinated animals showed marked agglutinin development during approximately two months following vaccination. Thereafter, there was a general recession in titers until the time of challenge when they ranged from negative at 1 : 25 to incomplete at 1 : 200. This delay in titer recession probably was due to the age of the heifers at time of vaccination. Agglutinin titers of the nonvaccinated heifers never exceded incomplete at 1 : 25 in any test made prior to their Brucella challenge.

Following Brucella exposure, 3 of the 6 M vaccinated animals (11, 16, 20) developed reactor titers which were maintained to the termination of their gestations. The other heifers of this group showed titers ranging from negative at 1 : 25 to incomplete at 1 : 50. Some titer increase was observed in the strain 19 vaccinated animals following exposure; however, the titers at time of calving were at most but slightly increased above that noted at the time of exposure. Heifer 17 showed a titer of 1 : 200 at calving which had increased to incomplete at 1 : 3200 approximately two months later.

Five of the 6 heifers in the nonvaccinated group developed titers which at calving ranged between incomplete at 1 : 400 to incomplete at 1 : 6400. The remaining heifer (21) gave a titer of incomplete at 1 : 100 in the first test following exposure with the titer receding to not greater than incomplete at 1 : 25 thereafter.

The outcome of gestations and the results of attempted recovery of *Br. abortus* following Brucella exposure are presented in table 2. Two of the 6 M vaccinated heifers (16, 20) aborted and 1 (11) had a premature calf that lived. *Brucella abortus* was recovered from colostrum and/or vaginal fluid of each. Three heifers (5, 14, 15) had normal calvings and *Br. abortus* was not recovered.

Each of the 5 strain 19 vaccinated heifers had normal calvings; however, *Br. abortus* was recovered from vaginal fluid of heifer 17. This heifer was the only one of the strain 19 group that had shown definite agglutination reactions following Brucella challenge. Four of the 6 nonvaccinated heifers (1, 2, 3, 8) aborted and 1 (4) had a premature calf. *Brucella abortus* recoveries were obtained from each. Heifer 18 had a normal calving and efforts to recover *Br. abortus* from her colostrum and vaginal fluids were unsuccessful.

DISCUSSION

Evaluation of the establishment of infection in the strain 19 vaccinated animals on the basis of agglutinin development following Brucella challenge of the heifers was somewhat uncertain due to the existing titers, probably the result of vaccine administration, that were present at the time of exposure. However, the only heifer in this group from which *Br. abortus* was recovered showed a definite reactor titer. All other Brucella exposed heifers that developed and maintained reactor titers either

TABLE 2—Results from Brucella Abortus Challenge of Pregnant Heifers

Group and No.	Approx. age at vaccination (mo.)	Gestation at exposure (days)	Calving and days in gestation	Br. abortus recoveries cultural method only		
				fet. stom.	colostrum	vag. sw.
M-Vac.						
no. 5	11	173	N—271	.....	neg.	neg.
11	15	162	P—263	.....	pos.	pos.
14	8	113	N—276	.....	neg.	neg.
15	8	130	N—289	.....	neg.	neg
16	11	124	A—212	No exam.	pos.	pos.
20	8	170	A—236	No exam.	pos.	pos.
19-Vac.						
no. 6	10	188	N—273	.....	neg.	neg.
7	10	137	N—277	.....	neg.	neg.
17	10	144	N—272	.....	neg.	pos.
19	8	185	N—275	.....	neg.	neg.
21	10	186	N—271	.....	neg.	neg.
No Vac.						
no. 1	12	195	A—244	pos.	pos.	pos.
2	11	130	A—202	pos.	pos.	pos.
3	12	139	A—213	pos.	pos.	pos.
4	11	185	P—251	.....	neg.	pos.
8	11	158	A—228	pos.	pos.	pos.
18	11	117	N—273	.....	neg.	neg.

N = negative; P = premature, calf alive; A = aborted, calf dead.

aborted or had premature calves and *Br. abortus* was recovered in each instance.

The average gestation at the time of Brucella challenge of the three groups of heifers was 145.4 days in the M vaccinated animals; 168 days in the strain 19 group, and 154 days in the nonvaccinated group. It appears unlikely that this variation was a factor of significance in the outcome of infection, since the average gestation at time of exposure of the nonvaccinated heifers that became infected was 161.4 days.

Based on the recovery of *Br. abortus* from the heifers or their fetuses at the time of calving, infection was established in 50 per cent of the M vaccinated animals, 20 per cent of the strain 19 group, and 83.3 per cent of the nonvaccinated controls.

These results give evidence as to the virulence of the exposure culture and the adequacy of the dosage employed under the conditions of this experiment. It is likewise evident that the exposure was not so great as to entirely overcome the protective value of the vaccines used. Since neither vaccine gave 100 per cent protection to the challenging test and if the 16.7 per cent failure of infection in the group of nonvaccinated heifers may be accepted as representing the rate of normal resistance for each of the groups under similar condition of exposure, the protective values would be 33.3 per cent for the M and 63.3 per cent for the strain 19 vaccines. Under conditions of this experiment, the protection by strain 19 vaccines against experimental challenge was greater than that afforded by the M vaccine. However, since the M vaccine does not produce the high or persistent blood titers frequently observed following the use of strain 19, especially in adult cattle, it may have an additional value which is not appraised by the results of the present experiment.

Summary

A test of the protective value of Huddleson M and BAI strain 19 vaccines administered to young dairy heifers which later during the four to six months of gestation were experimentally exposed to virulent *Brucella abortus* organism is reported.

The experimental heifers were selected from a Brucella agglutination-negative herd and ranged from 8 to 15 months in age at the time of vaccine administration.

One group of these heifers was given Huddleson M vaccine, another received strain 19 vaccine, and a third group receiving no vaccine was used as controls.

During the four to six months of pregnancy, and approximately sixteen months following vaccine administration, each pregnant heifer was subjected to experimental exposure by conjunctival sac instillation of 750,000 viable organisms of a virulent culture *Br. abortus*.

The protective value of the vaccines was assessed on the basis of recovery of *Br. abortus* by cultural methods from specimen material obtained from each heifer or her aborted fetus at or near the time of calving.

Brucella abortus cultures were obtained from 5 (83.3%) of 6 nonvaccinated controls, 3 (50%) of 6 M vaccinated animals and 1 (20%) of 5 strain 19 vaccinated animals.

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Studies on Corpus Luteum Ablation and Progesterone Replacement Therapy During Pregnancy In the Cow

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Literature

Removal of the corpus luteum during early pregnancy will terminate pregnancy in the cow according to Hess[11] and Schmaltz.[20] Sartoris[19] found that removal of the ovary containing the corpus luteum or both ovaries in cows more than six months pregnant resulted in abortion in all cases. The corpora lutea have been found necessary for the maintenance of pregnancy throughout most if not all of gestation in the rat,[13] the goat,[6] and the rabbit.[4] The ovaries or corpora lutea can be removed before the end of gestation without interrupting pregnancy in sheep,[3] monkey,[5] guinea pig,[14] dog and cat,[5] mare,[8] and woman.[1]

Raeside and Turner[18] and Uren and Raeside[21] reported an experiment with heifers where daily injections of 25, 50, or 75 mg. of "pure" progesterone in olive oil after removal of the corpus luteum between the thirty-sixth and seventy-sixth day of pregnancy failed to maintain pregnancy to term. Removal of the corpus luteum on the 205th day of pregnancy without subsequent progesterone injections was followed by delivery of a live calf on the 273rd day of pregnancy.

Meites *et al.*[17] were able to maintain pregnancy in 3 goats after removal of the corpus luteum on the one hundredth day of pregnancy by administering 25 mg. of progesterone daily from the ninety-ninth to the 149th day of pregnancy. Parturition occurred on the 151st day of gestation.

The possibility that a poorly functioning corpus luteum might lead to resorption or abortion of the fetus prompted this investigation to determine whether pregnancy could be maintained in the cow by administering crystalline progesterone in sesame oil after surgical removal of the corpus luteum at approximately the sixtieth day of pregnancy. Provided pregnancy could be maintained by replacement therapy, the study sought to determine the necessary amounts of progesterone. Finally, the study sought to determine whether exogenous progesterone is necessary throughout pregnancy.

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Dr. McDonald is a research fellow of the American Veterinary Medical Association.

Materials and Methods

Eighteen mature grade Holstein-Friesian cows averaging 1,200 lb. in body weight were used for this study. Approximately one-half had calved previously, but none were lactating during the experiment. Serum-agglutination tests for brucellosis were negative before and during the experiment. The husbandry of these animals was excellent. They were kept on a hay and grain ration throughout the year, and were bred by natural service. Breeding was begun in September, 1950, and was completed by April, 1951. Day one of pregnancy was considered the day following insemination.

Pregnancy was confirmed by palpation of the uterus through the rectum. At approximately the sixtieth day of pregnancy, each test animal was subjected to surgical removal of the corpus luteum. The corpora from the first 3 animals were removed through an incision in the abdominal wall in the paralumbar fossa area. Subsequent ablations of corpora lutea in other animals were through incisions in the anterodorsal walls of the vaginas.[16] In all cases, the corpus luteum was completely removed from the body cavity. The ovaries were examined for the presence of corpora lutea which may have resulted from multiple ovulations. Histological sections of most of the extirpated corpora lutea were prepared and examined to confirm completeness of their removal.

The progesterone used for replacement therapy was U. S. Pharmacopoeia, Melting Point 128 C., in sesame oil, 10 mg. of progesterone per milliliter. Intramuscular injections were given in the gluteal region, using a 16-gauge, 3-in. needle, once daily in the late afternoon.

Results

Control animals were used in this investigation, even though clinical evidence has shown that corpus luteum removal during early pregnancy usually is followed by abortion. Five cows were subjected to corpus luteum ablation between the ninety-second and the 163rd days of the gesta-

tion period (fig. 3) without subsequent progesterone injections. All pregnancies terminated promptly; abortion of the intact fetuses occurred between four and eleven days after surgery, with the exception of cow 226 which discharged a macerated fetus.

The Maintenance of Pregnancy after Corpus Luteum Removal.—The first objective was to determine whether pregnancy could be maintained after the removal of the corpus luteum. One hundred milligrams of crystalline progesterone in sesame oil was administered intramuscularly daily to 8 animals beginning on the day of corpus removal. Seven of these 8 animals delivered live calves (fig. 1). The fetus of cow 946, however, became mummified about the 170th day of pregnancy, injections were discontinued on the 182nd day of pregnancy, and abortion of the mummified fetus occurred between 187 and 194 days after conception.

The Quantity of Progesterone in Sesame Oil Needed to Maintain Pregnancy.—The next objective of the study was to determine the minimum daily amount of progesterone necessary to maintain pregnancy. After the first group of animals had maintained pregnancy for one month on a dosage of 100 mg. of progesterone, 6 other cows were given a dosage of 75 mg. of progesterone in sesame oil daily, beginning on the day of corpus luteum removal, which was approximately the sixtieth day of pregnancy. Only two pregnancies were maintained (fig. 2). Cow 037 delivered a live calf. Cow 392 maintained pregnancy during the forty-three-day interval while receiving 75 mg. of progesterone daily. However, after injections were discontinued on the 102nd day of this animal's pregnancy, as part of another study, a uterine discharge of the macerated fetus occurred during the interval of the 125th to 138th days of pregnancy. Cow 944 failed to maintain pregnancy during two pregnancies (trials 944 and 944a) on the lower dosage. Likewise, cow 465 failed to maintain pregnancy during two pregnancies (trials 465 and 465a) while receiving the lower dosage of progesterone.

Discontinuance of Injections.—The next trial was designed to determine whether injections were needed until the normal termination of pregnancy. Progesterone injections were discontinued on the 237th

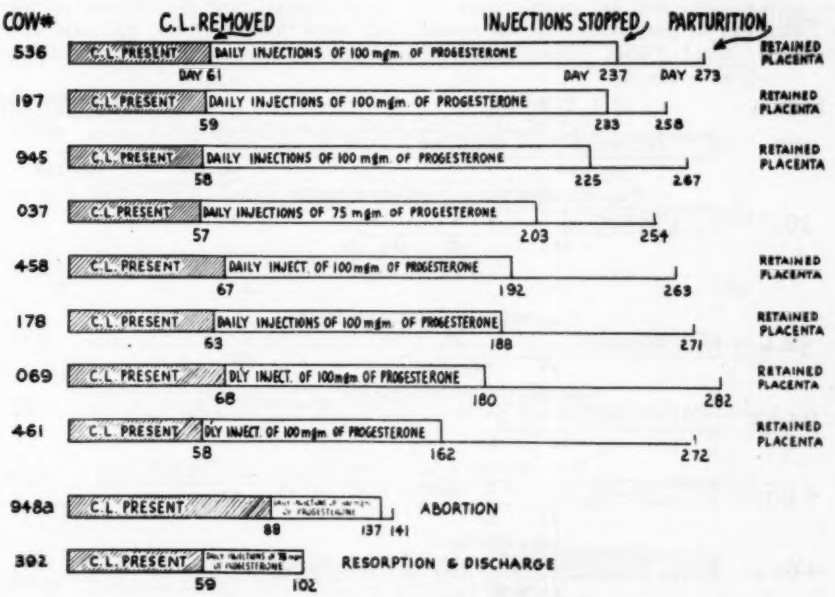

Fig. 1—Corpus luteum removal followed by daily intra-muscular injections of 100 mg. of progesterone in 10 ml. of sesame oil maintained pregnancy in 7 of 8 animals.

day of pregnancy in cow 536 (fig. 4), and a live calf was delivered on the 273rd day. The length of time that progesterone injections were given to each subsequent cow was progressively shortened until, in cow 461, pregnancy was maintained when injections were discontinued on the 162nd day. This cow delivered a live calf on the 272nd day. Lactation began in cows 461 and 069 within one week after discontinuance of progesterone injections. Cow 948a was not given injections of progesterone after the 137th day, and an intact fetus was aborted four days later. Similarly, the pregnancy of cow 392 was terminated after cessation of injections of progesterone on the 102nd day. Lactation also began in cows 948a and 392 within one week after discontinuance of progesterone injections.

Clark[3] cited evidence to indicate that removal of the corpus luteum before the thirty-fifth day of pregnancy may be followed by ovulation and corpus luteum formation without interruption of the existing pregnancy. Cow 466 was used to investigate the possibility that ovulation and development of functional luteal tissue might have occurred after removal of the corpus luteum at the sixty-third day of pregnancy. Histological examination of the ovaries of this animal, obtained by ovariectomy on the 203rd day of pregnancy, did not reveal functional luteal tissue. The length of gestation of those cows which delivered live calves showed considerable variation (fig. 4). There was a range from 254 to 282 days after breeding with a mean of 267.5.

Retained placentas occurred in 7 of 8 cows that delivered live calves. These 8 cows had been subjected to removal of the corpus luteum (fig. 4) at approximately the sixtieth day of pregnancy and had received injections of progesterone for varying lengths of time. None were receiving injections at the time of parturition. Histological examination of sections from the maternal-fetal placental attachment of 1 cow did not reveal placentitis. Manual removal of the placentas two or three days after calving was extremely difficult in all cases and a mild endometritis followed.

DISCUSSION

The necessity of progesterone or progesterone-like substances for the maintenance of early pregnancy is substantiated by early termination of pregnancy following the removal of the corpus luteum from 5 cows between the ninety-second and 163rd day of pregnancy. The fetus apparently dies within hours after the level of progesterone in the blood drops. Courrier[5] demonstrated that the ovaries of the rabbit were necessary to maintain the life of the fetus or placental membrane *in*

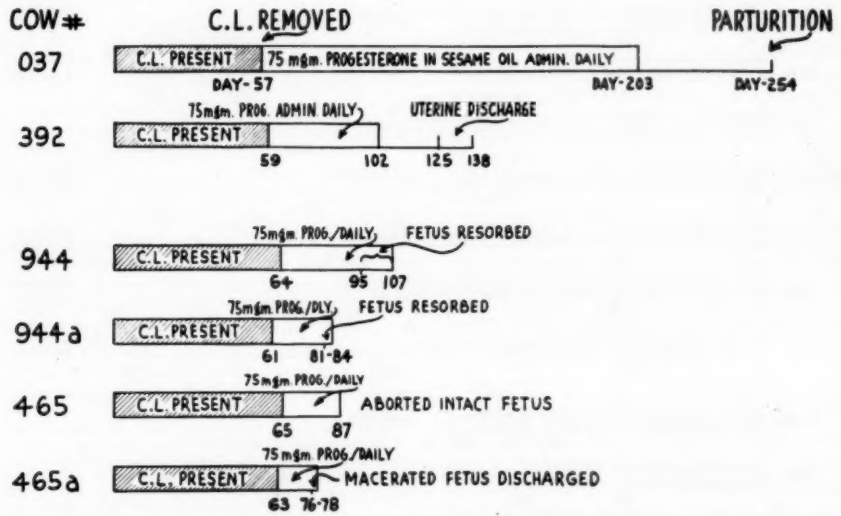

Fig. 2—Corpus luteum removal followed by daily intra-muscular injections of 75 mg. of progesterone in 10 ml. of sesame oil maintained pregnancy in two of six pregnancies.

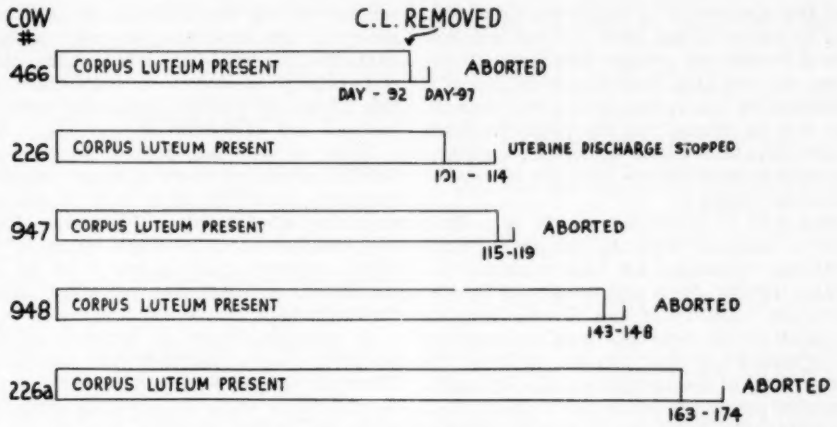

Fig. 3—Corpus luteum removal between ninety-two and 163 days after conception resulted in abortion.

utero, but were not necessary for the extra-uterine growth of a fetus. If this condition exists in the cow, then the rapid termination of pregnancy in these experimental animals can be postulated to be due to the lack of progesterone and its ability to maintain the uterus in a state compatible with pregnancy.

The absorption rate of the sesame oil and progesterone is unknown. Apparently, the blood level of progesterone did not fall below the critical level for the life of the fetus during the interval between injections in the 7 cows that received 100 mg. Additional work[16] with progesterone microcrystals in an aqueous solution* indicates

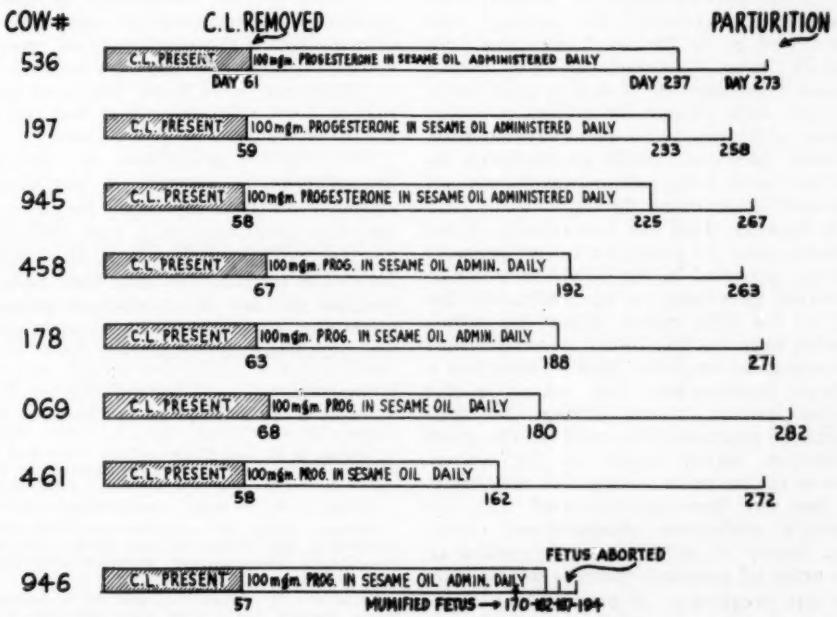

Fig. 4—Discontinuing progesterone injections as early as day 162 was not followed by abortion. Discontinuing injections on day 137 was followed by abortion.

that the quantity of progesterone necessary may be nearer 50 mg. than 100 mg. per day. The difference in dosage requirements between the two preparations may be due to a difference in absorption rates. No explanation can be offered for the mummification of the fetus of cow 946, since the pregnancy had been maintained for 113 days by the replacement therapy.

The data available on the 75 mg. level seem to indicate that the amount of progesterone necessary for fetal viability increases slowly. This was evidenced by the continued viability of the fetuses for ten to eighteen days on this level in contrast to expulsion of the fetuses in four to eleven days in the control group. The data available on this level do not indicate that a relationship exists in which the relative degree of deficiency of progesterone is reflected by whether the fetus is aborted or resorbed as suggested by Raeside and Turner.[18]

The occurrence of lactation during the maintenance of pregnancy after early discontinuance of progesterone injections in cows 069 and 461 (fig. 4) is in contrast to the occurrence of lactation with the termination of pregnancy in cows 948a and 392 after discontinuance of progesterone injections. Apparently the ovarian hormone level in the former 2 cows was high enough after discontinuance of progesterone injections to maintain pregnancy, but not high enough to inhibit pituitary release of lactogenic hormone. Termination of early pregnancy while progesterone injections were being administered was not followed by lactation (fig. 2).

It appears from the subsequent earlier interruptions of progesterone replacement therapy reported in fig. 4 that in order to maintain pregnancy to approximately the end of the fifth month either the corpus luteum must be left intact or replacement therapy must be given. Dukes[7] described a cellular degeneration that occurs in the bovine corpus luteum during the fifth month of pregnancy, but without the gross involution which occurs in the corpus luteum of the mare during late pregnancy. It has not been demonstrated that the placenta elaborates progesterone except that Henry *et al.*[10] found pregnandiol in the urine of pregnant castrate women during late pregnancy. If progesterone is essential to the maintenance of late pregnancy in the cow then one may postulate that the placenta or some other tissue elaborates progesterone or a substance that has progesterone-like properties after injections are discontinued.

Jafar *et al.*[12] found in a study of 384 Holstein-Friesian cows a mean length of gestation of 278.2 days with a standard deviation of 4.8 days. The mean length of gestation of 267.5 days found in this study suggests that a low level of progesterone is conducive to early uterine contractions.

A hormonal-cause of retained placenta has not been demonstrated previously. There is the possibility that these experimental cows were maintaining pregnancy with an altered balance of ovarian hormones. Experimentally-induced retention of the placenta warrants further investigation.

*"Repositol," Pitman-Moore Company.

SUMMARY

Control cows in which the corpora lutea were removed between the ninety-second and 163rd day of pregnancy aborted promptly in the absence of progesterone replacement therapy.

Pregnancy was maintained by the injection of progesterone in eight of nine cows in which the corpora lutea were removed at approximately the sixtieth day of pregnancy. The mean length of gestation of these cows was short and retained placentae occurred in 7 of 8 cows.

One hundred milligrams of crystalline progesterone in sesame oil administered intramuscularly daily was necessary to maintain pregnancy in a high percentage of the experimental cows. In cows in which the corpora lutea had been removed, abortion did not occur when progesterone injections were discontinued as early as the 162nd day of pregnancy.

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Correction

In table 3 and the script on page 147 of the April, 1952, issue of the *American Journal of Veterinary Research*, the units per milliliter of penicillin should read 100, 200, 300, and 400, and of aureomycin 25, 50, 75, and 100. These errors appear in the article "Effect of Antibiotics in Diluted Bull Semen on *Vibrio Fetus*" by W. N. Plastridge and H. L. Easterbrooks.

Relative Efficacy of Various Methods of Obtaining Preputial Samples for Diagnosis of Trichomoniasis in Bulls

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THE FINDING of *Trichomonas fetus* in preputial samples from bulls either by direct microscopic examination or by culture is at present the most widely used method of diagnosis for trichomoniasis.

Hammond, Bishop, Jeffs, and Binns[1] found that 90 per cent of 241 preputial samples from 6 known infected bulls were positive by direct microscopic examination. However, Schneider[2] reported that of 19 bulls found infected with trichomoniasis 11 were negative by direct examination and were diagnosed as positive only by the use of cultures. There is lack of agreement among investigators as to the best method of obtaining preputial samples. Three methods are most important. The pipette method, as described by Hammond and Bartlett,[3] involves the use of a special pipette for obtaining a sample of the preputial fluid from the area of the glans penis. The swab method, as described by Morgan,[4] involves absorption of preputial fluid by a cotton swab wrapped arcund the end of a metal rod. The douche method, as described by Schneider,[2] involves introducing 200 cc. of saline solution into the sheath, external manipulation of the penis and sheath, drawing off the fluid and centrifuging. Hess[5] recommends the induction of sexual excitement in the bull immediately before sampling. It was the purpose of this study to determine which of these methods of obtaining preputial samples is the most efficacious.

METHODS

Eight bulls infected with trichomoniasis were used in this study, which was conducted from June, 1950, to February, 1952. Usually, 2 to 3 bulls were studied simultaneously. In the first part of the study, each of 7 bulls was sampled on alternate days with the pipette and swab methods and, in the second part of the study, each of 3 bulls was sampled similarly with the douche and pipette methods. In all methods sterile equipment was used, and in obtaining each sample an attempt was made to collect material from the surface of the glans penis and adjacent prepuce. During the sampling procedure the bull was usually held at the head by an assistant.

In the pipette method, a pipette about 55 cm. long made of 8-mm. glass tubing with a 4-mm. opening at the end was used. Four-ounce rubber bulbs were used with the pipettes. The method of sampling was similar to that already described.[1] After collection, the sample was put into a graduated centrifuge tube, and the volume was recorded.

In the swab method, an absorbent cotton swab about 6 to 8 cm. long and 1 to 2 cm. in diameter was fastened to the end of a 60-cm. steel wire about 4 mm. in diameter. A speculum was inserted into the orifice of the sheath prior to introducing the swab in order to reduce contamination of the sample. The swab was inserted as far as possible, then moved forward and backward and rotated around the glans penis. After removal from the sheath, the swab was inserted into a centrifuge tube containing enough (usually 0.5 to 1.0 cc.) saline solution to saturate the swab. Then the swab was rotated and pressed against the wall of the tube until as much fluid as possible was squeezed out. Rarely the swab was saturated upon removal from the sheath and no addition of saline solution was required.

In the douche method, a pipette about 15 cm. in length and 8 mm. in diameter was used with a 4-oz. rubber bulb to introduce approximately 200 cc. of sterile normal saline solution into the sheath. It was necessary to insert the pipette into the sheath twice, because the bulbs were inadequate in size. Prior to this, the orifice was thoroughly washed with warm water. After introducing the fluid, one hand maintained closure of the orifice while the other hand was used to manipulate the glans penis through the sheath, and to a lesser extent the other regions of the prepuce. Then the fluid was allowed to drain into a 600-cc. beaker. This fluid was centrifuged ten minutes at 2,000 revolutions per minute (r.p.m.) in 50-cc. tubes. After decanting the supernatant fluid, the remaining material was put into a 15-cc. graduated tube and centrifuged again for ten minutes at 2,000 r.p.m. After pouring off most

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of the supernatant fluid, the sediment was mixed with the remaining fluid and the total recorded.

Microscopic examination of the samples was conducted within a half hour of collection in most cases. In examining the sample, it was first thoroughly mixed in the centrifuge tube by drawing it into a pipette and expelling it a number of times. Then a portion of the sample was removed with the pipette and examined on a hemacytometer. In the majority of cases, the concentration of trichomonads was sufficient so that only those in the ruled areas were counted, but in some cases it was necessary to include the entire area under the cover slip. In cases of negative samples, additional portions were checked from the bottom of the tube after the samples had been allowed to undergo sedimentation.

In order to determine whether the concentration of trichomonads in the preputial fluid is affected by sexual excitement of the bull, five pipette samples were obtained from each of 2 bulls immediately after erection of the penis induced by teasing with a cow, and five samples were obtained on alternate days from these bulls without prior sexual excitement. The trial was repeated using the douche method instead of the pipette method on 2 bulls. In each of 3 bulls, pipette samples were obtained on three or four occasions immediately before and after sexual excitement of the bull.

RESULTS

The results of the study of the pipette and swab methods are presented in table 1. From the 7 bulls, 63 samples were obtained with the pipette method, and 62 were obtained with the swab method. The average number of trichomonads in thousands per cubic centimeters in the positive pipette samples was 20.6, while in the swab method it was 4.2. Therefore, the average concentration in the pipette method was about five times as great as in the swab method. In only 1 bull (6) did the concentration in the two methods approach equality; in all the rest it was at least twice as great in the pipette method. There was less variation in the average concentration of trichomonads among the bulls in samples obtained by the swab method; the range in average concentration was from 0.8 to 7.8 in thousands per cubic centimeters, while in the pipette method it was 4.2 to 74.6. The percentage of negative samples was not markedly different in the two methods (6.3 in the pipette method as compared with 8.1 in the swab method), but only 1 bull (7) had negative samples with the pipette method while 2 bulls (7 and 3) showed negative samples with the swab method.

The results of the study of pipette and douche methods are shown in table 2. From the 3 bulls a total of 119 samples was obtained with each method. The average number of trichomonads in thousands per cubic centimeters in positive samples obtained by the pipette method was 7.1; the corresponding figure for the douche method was

TABLE 1—Comparison of Results of Pipette and Swab Methods of Obtaining Preputial Samples

	Pipette					Swab				
Bull No.	No. of samples	Ave. cc. of samples	No. of neg. samples	Percent of neg. samples	Ave. No. *T. fetus* in thous. per cc.	No. of samples	Ave. cc. of samples	No. of neg. samples	Percent of neg. samples	Ave. No. *T fetus* in thous. per cc.
1	8	0.24	0	0	8.1	8	0.26	0	0	3.3
2	8	0.28	0	0	19.4	8	0.21	0	0	5.0
3	9	0.44	0	0	18.6	8	0.35	2	25	7.8*
4	4	0.25	0	0	74.6	4	0.44	0	0	5.5
5	2	0.73	0	0	14.1	2	0.35	0	0	3.6
6	3	0.67	0	0	4.1	3	0.51	0	0	3.5
7	29	0.34	4	14	5.0*	29	0.35	3	10	0.8*
Total	63	...	4	..	....	62	...	5	..	...
Average	9	0.42	0.6	6.3	20.6*	8.9	0.35	0.7	8.1	4.2*

*Average number in positive samples.

13.5. In 1 bull (5) the number was about three times greater in the douche method; however, in another bull (8) the average concentration was about twice as high in the pipette method as in the douche method and in the third bull (7) the average concentration was nearly equal in the two methods. Therefore, the two methods do not appear to differ markedly with respect to concentration of trichomonads in positive samples. The average percentage of negative samples was 34.4 for the pipette method and 18.5 for the douche method. The number of negative samples was higher for each bull in the pipette method, although in 1 bull (8) it was only slightly higher. Since the rate of occurrence of negative samples was lower in the douche method, this method was more efficacious than the pipette method in demonstrating trichomonads in this study.

In bull 8 in which the percentage of negative samples was relatively high, the samples usually contained much pus, apparently as a result of bacterial infection. Cultures from the sheath of this bull showed *Micrococcus pyogenes* and *Pseudomonas aeruginosa*.

The results of the tests of influence of sexual excitement on concentration of trichomonads in the preputial samples are shown in table 3. In the total of 30 samples taken without previous sexual excitement of the bull, there was no marked difference in either number of negative samples or average concentrations of trichomonads as compared with the 30 samples taken after sexual excitement. In the samples taken on alternate days, only one negative sample occurred and it was taken by the pipette method without prior excitement. In 1 bull (5) there was a distinctly higher average concentration of trichomonads in the samples taken after excitement.

In the samples taken with the pipette method immediately before and immediately after sexual excitement of the bull there were 2 cases, in bulls 6 and 7, in which the first sample was negative while the corresponding second sample was positive. Except for these 2 cases the concentration of trichomonads was lower in the samples taken after excitement than in the corresponding samples taken before excitement.

DISCUSSION

In the pipette method, fluid from the areas of the preputial cavity most likely to harbor trichomonads is obtained and examined directly without dilution. In the swab method, fluid and cellular material from the same areas are absorbed by the swab and this must then be pressed out of the swab, usually involving dilution of the sample with saline solution. It is likely that some trichomonads with other cellular material remain in the swab, thus reducing the concentration in the sample. These factors are responsible, at least in part, for the marked difference in average concentration of trichomonads in samples obtained by the pipette and swab method, and for the less marked difference in occurrence of negative samples.

In the douche method, preputial fluid

TABLE 2—Comparison of Results of Pipette and Douche Methods of Obtaining Preputial Samples

	Pipette					Douche				
Bull No.	No. of samples	Ave. cc. of samples	No. of neg. samples	Percent of neg. samples	Ave. No. *T. fetus* in thous. per cc.	No. of samples	Ave. cc. of sediment	No. of neg. samples	Percent of neg. samples	Ave. No. *T. fetus* in thous. per cc.
5	29	0.53	8	27	9.1*	29	1.9	1	3	31.6*
7	48	0.41	11	23	7.0*	48	2.6	3	6	6.7*
8	42	0.58	22	52	5.1*	42	2.4	18	43	2.1*
Total	119	...	41	...	...	119	..	22	..	....
Average	39.7	0.51	13.7	34.4	7.1*	39.7	2.3	7.3	18.5	13.5*

*Average number in positive samples.

and cellular material is washed out of the sheath in saline solution and the cellular material is separated from the fluid by centrifuging. Since manipulation during the washing process is directed especially toward the region of the glans penis, presumably secretion and cellular material from this area is well represented in the sample. In this method, the quantity of sample is considerably greater than in the other methods (average 2.3 cc. sediment, as compared with 0.51 cc. fluid in the pipette method). This greater quantity of sample is usually not of any particular value in direct examination but may be advantageous in the use of cultures. The results of the present study indicate that there is no marked difference in average concentration of trichomonads obtained by the pipette and douche methods. This is an indication that the sediment obtained in the douche method has a concentration of trichomonads similar to that of the preputial fluid in the pipette method. However, the rate of occurrence of negative samples is lower in the douche method than in the pipette method, according to the present study. If conclusions can be drawn from such a limited number of bulls, the results indicate that the douche method is more efficacious in demonstration of trichomonads when these are at a low level of concentration in the preputial cavity. This may indicate that material containing trichomonads is washed out of recesses in the mucosa by the douche method.

The concentration of trichomonads in preputial samples is of importance in that it influences the accuracy and ease of diagnosis. The rate of occurrence of negative samples in an infected bull indicates the accuracy of the method involved. These two factors were considered of most importance in determining the relative efficacy of the methods under study.

Other factors, such as simplicity and amount of time required, are also of importance in selection of the best method for diagnosis. The pipette method is probably the simplest, since undiluted preputial fluid is directly examined. The disadvantages with this method is that the pipette is occasionally broken inside the prepuce with the possibility of causing wounds. This disadvantage could be avoided if a satisfactory pipette made of a plastic ma-

TABLE 3—Results of Tests of Influence of Prior Sexual Excitement of the Bull on Preputial Samples

Without prior excitement					With prior excitement				
Bull No.	No. of samples	Ave. cc. of fluid or sediment	No. of neg. samples	Ave. No. *T. fetus* in thous. per cc.	Bull No.	No. of samples	Ave. cc. of fluid or sediment	No. of neg. samples	Ave. No. *T. fetus* in thous. per cc.
Samples obtained on alternate days with pipette method									
7	5	0.59	0	4.80	8	5	0.84	0	3.00
8	5	0.64	1	0.49*	7	5	0.60	0	0.36
Average	5	0.61	..	2.60*	Average	5	0.72	..	1.68
Samples obtained on alternate days with douche method									
5	5	0.94	0	11.0	5	5	0.64	0	32.2
7	5	0.86	0	6.9	7	5	0.94	0	9.7
Average	5	0.90	..	8.9	Average	5	0.79	..	20.9
Samples obtained with pipette method immediately before and after excitement									
6	3	0.52	1	11.4*	6	3	0.72	1	6.2*
7	4	0.50	1	7.4*	7	4	0.64	1	0.7*
8	3	0.56	0	1.6	8	3	0.52	2	0.1*
Average	3.3	0.53	..	6.8*	..	3.3	0.63	..	2.3*
Total	30	...	3	...	..	30	...	4	...
Average	..	...	..	6.2*	..	...	...	..	7.4*

*Average number in positive samples.

terial were available. The swab method is less direct than the pipette method but may be preferred where a pipette is likely to be broken. Occasionally, the cotton swab may become detached from the steel wire while it is in the prepuce and when this happens it is difficult to obtain a good sample. The douche method requires more equipment and time than either of the other two methods. It is suitable only when the examination of the samples is to be made under laboratory conditions, while the other two methods may be used in the field.

The use of a bottle, flutter valve, and tubing for introducing the saline solution into the sheath and for receiving the sample as recommended by Harding,[6] and in a modified form by Florent,[7] is less advantageous than the rubber bulb and pipette, in the opinion of the authors. It was found that gravity flow of the saline solution into the sheath was somewhat slow and uncertain, as was the return flow into the bottle.

Hess[5] reported that the mucous fluid in the preputial cavity contains considerably more trichomonads after an infected bull undergoes an erection of the penis than before, because trichomonads, along with cellular detritus and secretion, are thereby pressed out of the recesses in the mucosa. He, therefore, uses a cow to bring about an erection of the bull immediately before collecting a preputial sample by a douche method.

The small number of tests on the influence of sexual excitement on the concentration of trichomonads in the preputial samples included in the present study indicates that in some bulls the concentration of trichomonads is sometimes increased by inducing an erection of the penis immediately before collecting the sample. However, the procedure involves additional time and difficulties in handling bulls, and its use is not ordinarily justified, in the opinion of the authors.

It is notable that the occurrence of negative samples was much less frequent in the first part of the study than in the second. In the first part of the study, the percentage of positive samples (93.7 with pipette, 91.4 with swab) compares favorably with that in a previous study[1] (90). The percentage of positive samples was considerably lower than this in the second part of the study (65.6 with pipette, 81.5 with douche). This difference may be explained in part by the presence of the bull (8) with pus-filled samples in the second part of the study. As in 1 bull in the previous study,[1] this condition was associated with a relatively high occurrence of negative samples.

During the latter part of the present study, cultures of most of the samples obtained by the pipette and douche method were made. The results of the work with cultures will be presented in a later paper.

SUMMARY

1) Seven bulls infected with bovine trichomoniasis were examined on alternate days with the pipette and swab methods of obtaining preputial samples. The concentration of *Trichomonas fetus* in the samples was estimated with the aid of a hemacytometer. The average number of trichomonads in thousands per cubic centimeter in 63 samples obtained by the pipette method was 20.6; for the 62 samples obtained by the swab method it was 4.2. The percentage of negative samples was 6.3 for the pipette method, with only 1 bull showing negative samples; for the swab method it was 8.1 with 2 bulls having negative samples.

2) Three infected bulls were examined on alternate days with the pipette and douche methods of obtaining preputial samples. The average number of trichomonads in thousands per cubic centimeter in 119 samples obtained by the pipette method was 7.1; for 119 samples obtained by douche method it was 13.5. However, in 1 of the bulls, the average concentration was markedly greater in the samples obtained by the pipette method, and in another bull the average concentration in the two methods was about equal. The average percentage of negative samples was 34.4 for the pipette method, while it was 18.5 for the douche method.

3) Limited tests indicate that induction of sexual excitement in the bull immediately prior to sampling is not ordinarily worthwhile in routine diagnosis.

4) The results of this study indicate that the douche method is more efficacious than the pipette or swab method for obtaining preputial samples in the diagnosis of bovine trichomoniasis, and should be the method of choice when samples can be

collected and examined in the laboratory. The results also indicate that the pipette method is more efficacious than the swab method and when samples must be examined in the field the pipette method is usually preferable.

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The Role of the Milking Process in the Intraherd Transmission of Q Fever Among Dairy Cattle

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IN EARLY experimental studies of Q fever in cattle, certain observations suggested that this disease in the bovine species was merely a local infection of the mammary gland.

In 1947, Parker *et al.*[1] exposed 4 heifers to large doses of *Coxiella burnetii* by intranasal, intravenous, and intravaginal inoculation, and by feeding contaminated bran. As determined by criteria available at that time, all 4 remained refractive to their respective routes of exposure. Following the recovery of *C. burnetii* from milk of naturally infected dairy cattle,[2] the disease was reproduced in 2 lactating cows by inoculation via the teat canal and by injecting the rickettsia (Nine Mile strain) into the mammary gland substance.[1] These cows developed complement-fixing antibodies against *C. burnetii* and continued to shed Q fever rickettsias in their milk for variable periods of time. Milk of uninoculated quarters remained free of *C. burnetii*. Furthermore, blood, urine, feces, and nasal secretions of these cows were tested on numerous occasions and determined to be noninfectious for guinea pigs.

In another experiment,[3, 4] which was planned primarily to study the symptomatology and pathology of bovine Q fever, 4 cows were inoculated with large doses of a California strain of *C. burnetii*, each via the teat canal of two quarters. All 4 cows became infected and shed rickettsias in milk of inoculated quarters. This massive exposure resulted in the production of a rickettsiemia during the first five days after exposure. Although infectious blood circulated through the tissues of uninoculated quarters, only one quarter became infected and shed rickettsias from the seventh to sixtieth day postinoculation. In this case, it was uncertain that this represented a localization from the blood stream, as the teat of this quarter was undoubtedly contaminated during milking; thus, entrance of rickettsias via the teat canal could not be excluded.

These cows were salughtered on the fifth, eleventh, twenty-second, and sixty-third days postinoculation. Tests of tissues revealed that *C. burnetii* persisted only for a brief period in organs other than the mammary gland and its regional lymphatics. The regularity with which infection could be produced by the injection of relatively large doses into the mammary gland, its brief persistence in extramammary tissues, and the exceptional occurrence of infection in uninoculated quarters of infected cows suggested that the disease might be a local infection of the mammary gland. From this evidence, it was postulated that natural infection was acquired by entrance of the organism through the teat canal. The milking process could be an important factor, as with streptococcal mastitis, in the dissemination of *C. burnetii* among dairy cattle.

This thesis was further supported by observations on naturally infected dairy cattle in California.[2, 5] In early tests of herds, serological evidence of infection was never found among bulls or heifers but only in the serum of mature cows with functional mammary glands. The absence of clinical illness and the failure to recover *C. burnetii* from blood, feces, and urine of cows shedding rickettsias in their milk suggested to these workers the probability of a local infection of the mammary gland with *C. burnetii*.[2]

In the late spring of 1949, an experiment was initiated to test the possibility of cow-to-cow spread through the milking process. After several months of repeated exposure during the milking process, it became apparent that transmission, if possible, would be difficult. Other experiments, concurrently conducted, yielded evidence indicating that the disease in cattle was more than a mere local infection of the mammary glands. Following intradermal inoculation of a pregnant cow,[6] *C. burnetii* was recovered from the milk and placenta. Exposure of another pregnant cow by the inhalation of an infectious aërosol spray resulted in a localization of infection in the placenta without an infection of the udder. Intradermal inoculation of calves resulted in demonstrable rickettsiemia and subsequent production of complement-fixing antibodies against *C. burnetii*. Further studies by Luoto and

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Huebner[7] in Southern California revealed that the placentas of naturally infected cattle frequently contained *C. burnetii*, some in high concentration. Thus, from accumulated evidence, the disease appeared to be that of a generalized infection with localization in the pregnant uterus and mammary gland.

Although the data discussed in the preceding paragraph indicated that Q fever in cattle was not a local infection of the mammary gland, the experiment was continued to evaluate the possibility of infection by contamination of the teats during the milking process. Since many serologically positive cows continue to shed Q fever rickettsias in their milk for extended periods, infectious milk represents a most constant source of infection. Furthermore, the fact that *C. burnetii* infection in cattle was not restricted to the udder did not preclude the teat canal serving as a portal of entry for this disease.

MATERIALS AND METHODS

In this experiment, 5 cows were exposed to *C. burnetii* by repeatedly dipping their teats in milk containing Q fever rickettsias. These cows were procured from two dairies located in the vicinity of Hamilton, Mont. Complement-fixation tests on cattle serums collected in this area have not revealed evidence of natural Q fever infections.[8] Prior to purchase, all cows were tested and determined to be free of tuberculosis, brucellosis, and Q fever. The herd from which cows 25, 26, and 27 were procured was tested and found to be free of these diseases. Cows 20 and 21 were taken from a herd which was free of Q fever and tuberculosis but contained a few suspicious reactors for brucellosis. These were animals that had been vaccinated as adults two years previously when brucellosis was diagnosed in the herd. At the time of purchase, the serum of cow 20 contained no agglutinins against Brucella, whereas a partial agglutination in a 1 : 50 dilution was detected in the serum of cow 21. During the experiment, the milk and placentas of these 2 cows were inoculated into guinea pigs and cultured for Brucella and no evidence of brucellosis was ever found. Data regarding age, breed, lactation, breeding, and calving dates, in relation to exposure periods are presented in table 1.

The anatomic features of the mammary glands of these cows were typical of the average grade dairy cow. Each had a medium-sized udder which was well attached and supported. The teat canals of cow 20 were rather large and permitted easy milking. The epithelium surrounding the teat orifices of this cow was roughened. With the exception of constricted teat canals on the fore quarters of cow 27, teats of the other cows were normal, with intact sphincters.

Cows were stanchioned in small individual isolation barns separated from each other by approximately 25 feet. Each barn was equipped with the cleaning tools, watering buckets, and other necessary equipment, to minimize the possibility of transferring infection from other cattle. Manure and bedding, which were re-

TABLE 1—Age, Breed, Lactation, Breeding and Calving Dates of 5 Cows in Relation to Exposure Periods

Cow No.	Breed	Age at start of exps.	1st calving date while on test	Ave. mo. milk production in lb.: weigh period	Breeding date impregnation occurred	2nd calving date while on test	Exposure period to pool 1 milk	Exposure period to pool 2 milk	Exposure period to milk of cow 12	Exposure period to milk of cow 24
20	Grade Holstein-Friesian	6 yr.	12/22/48	713 4/21/49 to 6/20/49	3/31/49 (1st service)	1/7/50	5/6/49 to 10/6/49	10/7/49 to 11/14/49 (dry period) 1/9/50 to 1/27/50	5/1/50 to 7/24/50	7/25/50 to 9/16/50
21	Grade Guernsey	4 yr.	4/5/49	986 4/21/49 to 6/20/49	9/21/49 (1st service)	6/25/50	5/6/49 to 10/6/49	10/7/49 to 1/27/50	6/25/50 to 7/24/50	7/25/50 to 9/16/50
25	Jersey	3 yr.	5/17/49	727 7/5/49 to 9/4/49	12/27/49 (1st service)	9/29/50	7/1/49 to 10/6/49	10/7/49 to 1/27/50	5/1/50 to 7/24/50	7/25/50 to 9/13/50
26	Jersey	3 yr.	3/4/49	653 4/21/49 to 6/20/49	12/26/49 (1st service)	10/6/50	5/6/49 to 10/6/49	10/7/49 to 1/27/50	5/1/50 to 7/24/50	7/25/50 to 9/13/50
27	Jersey	4 yr.	4/9/49	642 7/5/49 to 9/4/49	3/20/50 (4th service)	1/31/51	7/1/49 to 10/6/50	10/7/49 to 1/27/50	5/1/50 to 7/24/50	7/25/50 to 9/16/50

moved daily, were incinerated to prevent environmental contamination. Urine and floor washings were drained into underground sumps. Good quality alfalfa hay was fed free-choice; a grain supplement, consisting of equal parts of ground oats and barley, was fed according to milk production. All cows were hand-milked. Sterile rubber gloves were worn for personal protection, and also to preclude contamination of teats with *C. burnetii*, other than the measured amounts contained in the milk used for exposure.

For sake of clarity, the experiment will be discussed in two phases, namely, A and B. Throughout both phases, the following procedures were employed:

1) Just prior to experimental use, serums of these cows were again tested for complement-fixing antibodies against *C. burnetii* to insure the use of normal cattle; in addition, blood and milk of each cow were tested for the presence of *C. burnetii* by injection into guinea pigs.

2) At the morning milking, quarter milk specimens (fore milk) were collected in 2-oz. screw-top vials, three times weekly, on Monday, Wednesday, and Friday. Prior to the collection of milk specimens, the udder was washed with a 1 : 5,000 aqueous dilution of roccal (a mixture of alkyl-dimethyl benzyl ammonium chloride*) and then sponged free of excess antiseptic solution. At other milkings, the udders were not washed before milking. During phase B, after May 24, 1950, milk specimens were obtained without the preliminary washing of the udder with antiseptic solution. This was done to eliminate any possible factors which might interfere with the process of infection. Milk specimens were refrigerated (40 F.) immediately after collection and were processed within an hour. A portion of each specimen was frozen (—40 F.) in the event further testing was necessary.

3) All milk specimens were cultured for detection of bacterial pathogens by surface plating 0.1 ml. of milk on one-half of the surface of a bovine blood-agar plate and, also, a cell count was made on each quarter specimen, employing the Breed method.[9]

4) At weekly intervals, serums of these cows were tested for complement-fixing antibodies against *C. burnetii*, employing the method originally used by Plotz.[10]

5) Once weekly, individual quarter milk specimens were tested for the presence of *C. burnetii* by injecting each of 2 guinea pigs intraperitoneally with 5 ml. of milk. Approximately thirty days later, guinea pigs were bled and their serums were tested for complement-fixing antibodies against *C. burnetii*. The presence of complement-fixing antibodies in the serum of either or both guinea pigs indicated the presence of *C. burnetii* in the specimen injected or evidence of spontaneous infection. Duplicate frozen specimens of those which yielded positive results on initial injection were retested in 3 guinea pigs for confirmation. A negative confirmatory test indicated that the complement-fixing antibodies in serums of the guinea pigs of the original test resulted from spontaneous infection rather than from any *C. burnetii* contained in the specimens tested.

Phase A of this experiment extended from

*Roccal, Winthrop Chemical Co., New York, N. Y.

TABLE 2—Results of Inoculation of Cows 12 and 24 with Streptococcus Agalactiae and Coxiella Burnetii

				Infectivity of Milk									
				Days postinoculation									
		Quarter	M.I.G.P.D.†	3	10	17	24	31	45	59	73	87	92
Cow 12 4-yr.-old Guernsey	Inoculated with *C. burnetii* strain 16 April 4, 1950	RF	$1x10^7$	+	0	+	+	0	0	0	0	0	0
		RR	$1x10^6$	+	+	+	+	+	0	0	0	0	0
		LR	$1x10^5$	+	+	+	+	+	+	+	+	+	+
		LF	10	0	0	0	0	0	0	0	0	0	0
	Inoculated with *Str. agalactiae* April 27, 1950	RF	Strain K	N	N	N	N‡	P	P	P	P	P	P
		RR	Strain T 15	N	N	N	N‡	P	P	P	N*	N	N
				4	11	18	25	39	53	67			
Cow 24 3-yr.-old Holstein-Friesian	Inoculated with *C. burnetii*, strain 16 July 10, 1950	RF	$x10^7$	+	+	0	0	0	0	0			
		RR	$1x10^6$	0	+	0	0	0	0	0			
		LR	$1x10^5$	0	0	0	0	0	0	0			
		LF	10	0	0	0	0	0	0	0			
	Inoculated with *Str. agalactiae* July 24, 1950	RF	Strain K	N	N‡	P	P	P	P	P			
		RR	Strain T 15	N	N‡	P	P	P	P	P			

† Minimal infectious guinea pig doses. + = contained *C. burnetii* as determined by injection into guinea pigs. O = contained no *C. burnetii* as determined by injection into guinea pigs. P = *Str. agalactiae* isolated by blood-agar culture. N = No *Str. agalactiae* isolated. * = single treatment of 50,000 units of penicillin in 10 ml. saline. ‡ = inoculated with *Str. agalactiae*.

May 6, 1949, to Jan. 27, 1950. The periods of exposure for each cow are given in table 1. Cows were exposed five times a week, at the evening milking, by immersion of the teat ends in a 50-ml. beaker containing approximately 25 ml. of infectious milk. During the first part of the experiment the teats were dipped just prior to milking; however, after Sept. 13, 1949, teats were exposed both before and after milking to increase the chances of infection. Two pools of infectious milk were prepared from milk of cows experimentally infected with a California strain of *C. burnetii* (strain 16). This milk was frozen in 25-ml. quantities and thawed just prior to use. Blood-agar cultures of these two pools did not yield bacterial pathogens capable of causing mastitis. Based on an average of three different titrations, pool 1 contained approximately ten minimal infectious quinea pig (M.I.G.P.) doses / ml., whereas pool 2 contained approximately 1,000 M.I.G.P. doses / ml. (average of two titrations). Once weekly, guinea pigs were injected with the infectious pool milk which remained after exposure of the cows. All guinea pigs which received this pool milk regularly developed complement-fixing antibodies against *C. burnetii*.

Phase B of this experiment extended from May 1, 1950, to Sept. 16, 1950. About three months elapsed between phases A and B to permit any Q fever infection which may have been contracted from the latest exposures to develop and be recognized. Inasmuch as all 5 cows remained refractive to infection during phase A, an attempt was made to determine whether concurrent bacterial mastitis influenced susceptibility to Q fever and also to determine whether these particular cows were susceptible to streptococcic mastitis by this route.

Two cows, 12 and 24, which were experimentally infected with *C. burnetii* and *Streptococcus agalactiae*, served as a source of infectious milk. Both of these cows had been used in previous transmission experiments by other than the intramammary route. By all available criteria, neither had become infected. Just prior to inoculation, neither of these cows possessed complement-fixing antibodies against *C. burnetii*. Furthermore, preinoculation specimens of blood and milk were noninfectious for guinea pigs. Each cow was infected with *C. burnetii* by injecting 1 ml. of varying dilutions (*see* table 2) of a yolk sac culture (California Q fever, strain 16), via the teat canal, into the teat cistern of each quarter. Two strains of *Str. agalactiae*, which were procured from Dr. R. A. Packer, Iowa State College, Ames, were used to infect these cows. Fermentation reactions of both strains were typical.

Strain K formed acid from lactose, sucrose, and trehalose, but not from mannite, salicin, inulin, aesculin, sorbitol, and raffinose. Strain T15 was similar, with the exception that salicin was fermented. Both strains hydrolyzed sodium hippurate and acidified and coagulated litmus milk.

The right front quarter of each cow was infected by inoculation with 1 ml. of a 48-hour tryptose broth culture of strain K via the teat canal; the right rear quarter of each cow was infected in a similar manner with strain T15. Other pertinent data regarding the inoculation of these 2 cows are presented in table 2.

Milk from cow 12 was used for exposure of test cows from May 1 (27th day postinoculation

TABLE 3—Results of Tests for the Presence of C. burnetii in Milk Specimens from Five Exposed Cows

Phase A Exposure Period

Cow	RF		RR		LR		LF	
	Spont.* infection	Tests neg.	Spont. infection	Tests neg.	Spont. infection	Tests neg.	Spont. infection	Tests neg.
20	0	28	0	28	0	28	1	27
21	0	32	2	30	1	31	1	31
25	0	24	0	24	0	24	0	24
26	0	32	0	32	0	32	0	32
27	0	24	0	24	0	24	0	24

Phase B Exposure Period

Cow	RF & RR pooled		LR & LF pooled	
	Spont. infection	Tests neg.	Spont. infection	Tests neg.
20	0	12	1	11
21	0	9	0	9
25	1	11	0	12
26	0	12	0	12
27	0	12	0	12

*Specimens positive on initial injection but negative on retest.

with *C. burnetii*) to July 24, 1950, and milk from cow 24 was used from July 25 (15th day postinoculation with *C. burnetii*) to Sept. 16, 1950 (*see* table 2). Milk used for exposure, which consisted of a pool of aliquot quantities (100 ml.) from each quarter, was obtained at the same milkings the exposures were made. Cows were exposed in a manner and rate similar to that described for phase A. Once weekly, the pooled milk in which the teats of the 5 cows had been dipped was tested for the presence of *C. burnetii;* blood-agar cultures of these specimens were also made to obtain an approximation of the number of *Str. agalactiae* in the exposure milk. During the period of exposure to milk of cow 12, all exposure specimens from the beginning through the sixty-fifth day regularly contained *C. burnetii.* By titration it was determined that the milk used for exposure on May 18 and June 14, 1950, contained respectively 100 M.I.G.D./ml.** and 10 M.I.G.P.D./ml. The number of *Str. agalactiae* /ml. of exposure milk of cow 12 varied from 600 to 40,000 with a mean of 13,900. Only the first pooled milk specimen of cow 24, which was tested on the second day of this exposure period, was infectious for guinea pigs. The streptococcic content of the pooled exposure milk of cow 24 varied from 210 to 100,000/ml. with a mean of 35,000. Unfortunately, cow 24 ceased shedding *C. burnetii* shortly after inoculation. Since results of infectivity tests are not known until thirty-six to forty days after injection of specimens into guinea pigs, the milk of cow 24 was used for some time before its noninfectious nature was determined. During phase B, milk specimens from the exposed cows were tested for infectivity *(C. burnetii)* once every two weeks. Instead of testing individual quarter specimens, as was done in phase A, aliquot portions of the milk from the right front and right rear quarters and the left front and left rear quarters were pooled, and each pool injected into 2 guinea pigs.

**The rickettsial content of the highest dilution infecting 50 per cent or more of the guinea pigs is one minimal infectious guinea pig dose.

RESULTS

None of the 5 cows became infected with *C. burnetii.* Weekly complement-fixation tests of the serums of these cows collected during the periods of exposure were consistently negative. After exposure was terminated, cattle were bled once monthly for four months; none of these serums contained complement-fixing antibodies against Q fever. As noted in table 3, all tests for the presence of *C. burnetii* in the milk of these exposed cows were negative, except on seven occasions. Retest of frozen duplicate portions of these specimens failed to confirm the original positive results; hence, the first positive results probably represented spontaneous infections. On numerous occasions, frozen milk specimens of infected cows were retested and confirma-

TABLE 4—Leukocyte Counts of Quarter Milk Specimens of 5 Cows During Phase A Period of Exposure, Excluding Those Made the First Two Weeks and Last Two Weeks of Lactation

Cow No.	Quarter	No. counts below 200,000/ml.	No. counts of 200,000/ml. and greater	Periods of elevated leukocyte count	Bacterial pathogen respectively associated
20	RF	85	0	0	0
	RR	85	0	0	0
	LR	85	0	0	0
	LF	85	0	0	0
21	RF	110	0	0	0
	RR	110	0	0	0
	LR	110	0	0	0
	LF	110	0	0	0
25	RF	86	0	0	0
	RR	86	0	0	0
	LR	83	3	1st, 2nd	WHS, WHS
	LF	86	0	0	0
26	RF	110	0	0	0
	RR	110	0	0	0
	LR	110	0	0	0
	LF	110	0	0	0
27	RF	85	1	1st	WHS
	RR	85	1	1st	none
	LR	77	9	1st, 2nd	MHS, MHS
	LF	84	2	1st	MHS

NHS = Nonhemolytic staphlococcus. MHS = Mixed culture of hemolytic and nonhemolytic staphylococcus. HS = Hemolytic staphylococcus. WHS = Weakly hemolytic staphylococcus.

tory results were obtained. Therefore, if any of the seven specimens actually contained *C. burnetii,* they would have remained viable while frozen and caused Q fever infection when retested in guinea pigs.

The milk leukocyte count was considered a major criterion of the health of the mammary gland. During phase A, mammary glands of cows 20, 21, and 26 remained in excellent condition. Milk leukocyte counts of samples from cows 25 and 27 reflected mild attacks of staphylococcic mastitis on several occasions (*see* table 4). Recovery from these attacks was spontaneous without antibiotic therapy.

During phase B, cows 20, 25, and 27 acquired streptococcic mastitis, each in only one quarter (*see* table 5). *Streptococcus agalactiae* was first recovered from these cows on the thirty-first day, twenty-eighth day, and thirtieth day, respectively, after the first day of exposure.

During the first week of July, 1950, a tumor-like growth in the teat cistern of the right rear quarter of cow 20 became apparent. This increased in size so that it occluded the teat canal. By July 12, it became necessary to catheterize the teat to evacuate the quarter. The teat was cleansed and painted with merthiolate prior to catheterization. After this date, this teat was dipped in infectious milk only after completion of milking. Although it can not be compared with the more natural infections caused in cows 25 and 27, both *C. burnetii* and *Str. agalactiae* had equal opportunity to invade this quarter.

The streptococcic infections in the udders of cows 20 and 27 were permitted to progress and persisted throughout the period of exposure. However, the streptococcic infection in the left rear quarter of cow 25 resulted in such an acute mastitis on the tenth day of infection that treatment became necessary to prevent loss of the quarter. This occurred also in the case of the right rear quarter of cow 12 (*see* table 2); on the forty-second day after inoculation, an acute attack became so severe that treatment was necessary.

DISCUSSION

Since only 5 test cows were used, the significance of the preceding data is limited somewhat. Although natural conditions were not fully duplicated, the results of this experiment suggest that the teat canal is not a portal of entry for *C. burnetti* infection in dairy cattle. As these cows were stanchioned in isolation barns, they were not subjected to teat injuries and

TABLE 5—Leukocyte Counts of Quarter Milk Specimens of 5 Cows During Phase B Period of Exposure, Excluding Those Made the First Two Weeks and Last Two Weeks of Lactation

Cow No.	Quarter	No. counts below 200,000/ml.	No. counts of 200,000/ml. and greater	Periods of elevated leukocyte count	Bacterial pathogen respectively associated
20	RF	54	0	0	0
	RR	26	28	1st, 2nd, 3rd	HS, NHS, SA
	LR	54	0	0	0
	LF	54	0	0	0
21	RF	32	0	0	0
	RR	32	0	0	0
	LR	32	0	0	0
	LF	32	0	0	0
25	RF	51	1	1st	NHS
	RR	48	4	1st	MHS
	LR	29	23	1st, 2nd	HS, SA
	LF	40	12	1st	NHS
26	RF	52	0	0	0
	RR	52	0	0	0
	LR	52	0	0	0
	LF	52	0	0	0
27	RF	45	9	1st	NHS
	RR	7	47	1st, 2nd	MHS, SA
	LR	52	2	1st, 2nd	MHS, MHS
	LF	53	1	1st	NHS

NHS = Nonhemolytic staphylococcus. MHS = Mixed culture of hemolytic and nonhemolytic staphylococcus. HS = Hemolytic staphylococcus. SA = *Streptococcus agalactiae.*

trauma which may be predisposing factors to the infection by this route. However, the degree of exposure to which these cows were subjected probably exceeds that which would occur in hand-milked cows on naturally infected dairies. The infectious titer of the pools of milk used is about comparable to that of naturally infected milk which rarely contains an excess of 1,000 M.I.G.P.D./ml. (The milk of cows experimentally infected by extra-mammary routes has been found to contain a maximum of 100 M.I.G.P.D.) During phase B, conditions present in naturally infected herds were more closely simulated, since the daily milk of 2 infected cows was used. Thus, exposed cows were subjected to any possible marked fluctuation in the infectivity of exposure milk, which may not have been detectable by occasional titration. The amount of infectious milk deposited on the teats of these cows also exceeds that which may be carried from cow to cow on the hands of milkers.

These cows were exposed to *Str. agalactiae* in order to determine their susceptibility to a disease whose portal of entry is the teat canal, and also to ascertain whether a concurrent bacterial mastitis would influence susceptibility. Because of variables, such as age of cow, strain of *Str. agalactiae,* method of exposure, it is impossible to compare the results of this experiment with those of other investigators.[11-16] It is felt that the method of exposure to *Str. agalactiae* more closely duplicates natural conditions than many used by previous investigators. The fact that 3 of the 5 cows became infected with mastitis streptococci indicates that the anatomic and physiological features of these cows permitted such infection and, presumably, they could be susceptible also to infection with *C. burnetti* via this route.

During the first part of the experiment, the udders of the cows were washed with a 1:5,000 aqueous dilution of roccal before samples were taken. This was done to reduce bacterial contamination during sampling. At the time this procedure was discontinued, it was thought that residual roccal remaining on the teats could interfere with infection. Tests of the rickettsiacidal activity of roccal conducted at a later date[4] indicate that such interference was improbable. A 1:2,500 dilution of roccal in saline was mixed with an equal volume of a saline suspension of *C. burnetii* (1:500 dilution of a yolk sac culture of a California strain) and incubated at 40 and 98 F. At 98 F., *C. burnetii* survived for five hours but not for twenty-four hours, whereas, at 40 F. it survived for 112 hours, the longest exposure period tested. Under similar conditions, *C. burnetii* survived exposure to roccal in sterile skimmilk kept at both 40 and 98 F. for 112 hours, the longest period tested. These tests indicated that milk interferes with the activity of roccal. Thus, the rickettsiacidal activity of any residual roccal remaining on the teats would probably be insignificant.

The influence of pregnancy upon susceptibility of cattle to Q fever has not been determined. If stage of pregnancy affects susceptibility, adequate opportunity for such influence was afforded, since the 5 cows were exposed during all stages of pregnancy (*see* table 1). The long period of exposure also provided an opportunity for any possible predisposing factors, as yet unrecognized, to exert their influence in permitting infection via the teat canal. In spite of the regularity with which infection can be produced by injection of massive doses of *C. burnetii* into the udder, it is improbable that the teat canal is a portal of entry for natural Q fever infections in dairy cattle.

SUMMARY

Five cows, which were repeatedly exposed to *Coxiella burnetii* by dipping their teats in infectious milk, remained refractive to infection. These cows were then exposed to milk containing both *C. burnetii* and *Streptococcus agalactiae.* Three of the 5 contracted streptococcic mastitis, whereas all remained refractive to infection with Q fever rickettsias. These results suggest that natural Q fever infections in dairy cattle are not acquired by entrance of Q fever rickettsias through the teat canal.

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The Effect of Viomycin, 4:4′ Diamino Diphenyl Sulfone, and Other Agents on Mycobacterium Paratuberculosis

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Literature

Larsen *et al*[1] reported on *in vitro* studies of streptomycin, 4—aminosalicylic acid, promin, pentamidine isethionate, and stilbamidine diisethionate against *Mycobacterium paratuberculosis* and found streptomycin to inhibit development in low concentrations. Streptomycin was then administered to 4 cattle showing clinical evidence of paratuberculosis. These animals after showing a short period of improvement subsequently died of the disease or were destroyed as incurable.

Since that report was published, several new agents for the treatment of tuberculosis and leprosy have received considerable attention, and it was considered desirable to select several of these products for *in vitro* studies of their activity against *M. paratuberculosis*. In addition to the *in vitro* studies, 1 bovine animal in an advanced stage of Johne's disease was treated with one of the products. Studies were made on the following: viomycin, 4:4′ diamino diphenyl sulfone, and four different thiosemicarbazones (table 1). It is not within the scope of this paper to review the extensive literature on these agents so only a brief description is presented. 4:4′ diamino diphenyl sulfone, which is the parent nucleus of the sulfones, was selected because of the encouraging results obtained in the treatment of leprosy.[2-6] Thiosemicarbazone therapy of tuberculosis has been extensively utilized in Germany[7, 8] and has also received limited use in the treatment of leprosy.[9] Viomycin, a new antimicrobial agent isolated from broth cultures of *Streptomycin puniceus*,[10] was also included, since it has a pronounced suppressive effect on tuberculosis both *in vitro* and *in vivo*[10-13] and is of relatively low toxicity.[14, 15]

In Vitro Studies

These studies were made on a liquid medium prepared, sterilized, and inoculated with strain 18 *M. paratuberculosis* in the same manner as previously described.[1] Seven days after inoculation flasks with uniform growth were selected for the titrations. The thiosemicarbazones are not all readily soluble in aqueous solution so they were dissolved in the proportion of 0.5 Gm. of drug to 100 cc. of propylene glycol. This could be done since it was determined that propylene glycol had no effect on growing cultures of *M. paratuberculosis* when added in concentrations up to 3 per cent to several control flasks. This solution was then diluted with water to the desired concentrations. Each dilution of each agent tested was added to three culture flasks. Additional flasks containing equal growth were used as controls. All flasks received identical care and were kept side by side in the incubator at 37 C. As shown in table 1, the dilutions of the materials tested ranged from 400 μg. to 1 μg. per cubic centimeter of medium. All flasks were observed five times a week for one month after adding the agents being tested.

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Results

The results of the *in vitro* studies are shown in table 1. The thiosemicarbazones and 4:4′ diamino diphenyl sulfone prevented normal growth of *M. paratuberculosis* in all flasks when in concentrations of 200 μg. or more per cubic centimeter. They did not prevent growth when the medium contained 150 μg. or less per cubic centimeter, but part of the cultures continued to grow when the medium contained 175 μg. per cubic centimeter. Growth was not observed in flasks that contained 8.0 μg. or more of viomycin per cubic centimeter of medium, but was observed in one out of three flasks containing 6.0 μg. per cubic centimeter and in all flasks containing 4.0 μg. per cubic centimeter or less.

In Vivo Studies

Viomycin would have been the logical drug for *in vivo* tests since relatively small amounts prevented growth of *M. paratuberculosis*, but this antibiotic was not available in sufficient quantity for this purpose. Instead, plans were made to study the effect of 4:4′ diamino diphenyl sulfone since it had shown promise in treatment of leprosy[2, 3, 4, 6] even though it took relatively large amounts to prevent growth of *M. paratuberculosis* in cultures.

In collaboration with the School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn, a Jersey cow 5 years of age showing advanced clinical Johne's disease with severe diarrhea was used in this study. The diagnosis

had been confirmed by demonstrating small acid-fast bacilli indistinguishable from *M. paratuberculosis* in stained smears of rectal mucosa, and by obtaining a positive reaction from an intradermal injection of johnin.

The drug was administered in hard gelatin capsules daily over a period of four weeks. The daily dose per pound body weight was 35 mg. the first week, 22 mg. the second week, 10 mg. the third week, and 35 mg. the fourth week.

Results

The treated animal was observed closely during treatment. During the first two weeks, the feces became normal and the animal gained 55 lb. and showed marked improvement in general appearance. However, there was recurrence of diarrhea during the third week of treatment and continued loss of condition through the fourth week in spite of the increased dosage of the drug to its original level. The animal declined so rapidly that it was destroyed as incurable at the end of the fourth week. At necropsy, the intestine presented typical gross appearance of Johne's disease, and *M. paratuberculosis* was found in stained smears of the epithelium taken from the region of the ileocecal valve.

Discussion

As was the case with promin, 4-aminosalicylic acid, stilbamidine diisethionate, and pentamidine isethionate in previous studies,[1] the thiosemicarbazones and 4:4′ diamino diphenyl sulfone had to be added to mediums in relatively large quantities to prevent growth of *M. paratuberculosis*. Although the therapeutic results obtained with 4:4′ diamino diphenyl sulfone were not altogether encouraging, it should be borne in mind that this 1 animal was in a very advanced stage of the disease when treatment was initiated. Viomycin would appear to be the most active antibacterial agent for *M. paratuberculosis* of all those tested, since relatively small amounts of this antibiotic inhibit its growth *in vitro*. Since *in vitro* studies do not in themselves prove that these products are or are not of therapeutic value, *in vivo* tests should be made when possible, particularly on recently infected or less advanced clinical cases.

Summary

1) *In vitro* studies of 4:4′ diamino diphenyl sulfone, four thiosemicarbazones, and viomycin indicate that viomycin is the

TABLE I—Comparison of the Inhibitory Effect of Various Agents on Mycobacterium Paratuberculosis Cultures

Agent tested	No. of cultures used for each dilution	No. of cultures showing growth for each concentration (μg. per cc.) of agent tested											
		400.0	200.0	175.0	150.0	125.0	100.0	50.0	8.0	6.0	4.0	2.0	1.0
None (Control flasks)	6	6	6	6	6	6	6	6	6	6	6	6	6
p-formyl acetanilide, thiosemicarbazone (Myvizone)	3	0	0	2	3	3	3	3	..	..	..	3	3
p-hydroxy benzaldehyde, thiosemicarbazone	3	0	0	1	3	3	3	3	..	..	..	3	3
p-isobutoxy benzaldehyde, thiosemicarbazone	3	0	0	2	3	3	3	3	..	..	..	3	3
p-formyl acetanilide, 4-isobutyl 3-thiosemicarbazone	3	0	0	2	3	3	3	3	..	..	..	3	3
4:4′ diamino diphenyl sulfone	3	0	0	1	2	3	3	3	..	..	..	3	3
Viomycin	3	0	0	..	..	..	0	0	0	1	3	3	3

only one of these products that inhibits the growth of *Mycobacterium paratuberculosis* when used in low concentrations.

2) 4:4′ diamino diphenyl sulfone, administered in daily doses not exceeding 35 mg. per pound of body weight over a period of twenty-eight days, initially resulted in improvement of 1 cow in an advanced stage of Johne's disease; however, symptoms recurred two and one-half weeks after treatment was initiated and the animal was destroyed as incurable at the end of the twenty-eight-day treatment period.

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The Pathology of Bovine Mastitis Caused by Cryptococcus Neoformans

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THE CLINICAL ASPECTS and epizoötiology of an outbreak of mastitis in cows caused by *Cryptococcus neoformans* were given by Pounden, Amberson, and Jaeger;[10] identification of the organism was made by Emmons.[4] This paper is concerned with the study of the pathology of the disease in the same outbreak.

There are no other records of *C. neoformans* being identified as a cause of mastitis in any animal or human beings. Carter and Young,[2] however, isolated this organism from a bulk sample of milk derived from a tuberculin-tested herd of 25 cows in Scotland; no case of disease was subsequently detected in the herd when attempts were made to trace the source of the infection. Other types of yeasts and yeast-like organisms have been encountered in mastitis of cattle on several occasions, and references to these are given by Pounden *et al.*[10] and Emmons.[4] *Cryptococcus neoformans* is the causative agent of human cryptococcosis (torulosis), a disease process which frequently involves the central nervous system and the lungs. The same organism has been encountered as a pathogen occasionally in domestic animals.[11]

MATERIALS AND METHODS

Four of the cows studied were killed by shooting and bleeding and 5 by bleeding alone. The carcasses were eviscerated by a butcher, and all dissections of organs carried out by us. No organ in the body was exempt from examination, although that of the nervous system was limited in those cows which were shot. There were, however, no clinical findings to suggest that the nervous system had been involved in any case. The udder and lungs were removed whole, and were either incised at once, or were injected by formalin-saline into the natural channels and kept in the refrigerator for twenty-four hours before making whole vertico-transverse slices of the organs. Tissues for histological examination were fixed either in 10 per cent formalin-saline solution or in Zenker's fluid. Paraffin sections were prepared in the usual manner, and sections stained by hematoxylin-eosin, Ziehl-Neelsen, and the periodic acid (Schiff) leucofuchsin method. Numerous pieces were taken from affected and nonaffected parts of all quarters of the udders and, in some cows, all visceral and body lymph nodes from each side were sectioned separately. Cultures of various tissues were made for identification for the Cryptococcus organisms, as was described by Pounden *et al.*[10] and by Emmons,[4] and no positive cultures were obtained by them from any organ, other than the affected areas of udder and associated lymph nodes.

PATHOLOGY

The following descriptions deal with the pathology of the disease in a general way.

Fig. 1—Cryptococcosis, udder (cow 2589). Portion of affected udder and teat with abnormal, fleshy, coarse, lobulated appearance and some hemorrhage.

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Although there was a variation in the severity of changes from case to case, there was a fundamental similarity in both macroscopic and microscopic appearances, and the latter are unmistakably specific. The remarks are restricted to the significant changes in the udder and lymph nodes, and to focal cryptococcal lesions in the lungs of 1 cow.

Some other coincidental pathological conditions were observed in various organs, but they are irrelevant to the primary disease discussed. For example, in 1 cow there was a massive, saccular, nonspecific bronchiectasis (not cryptococcal) of almost the whole apex of one lung.

The hide was flayed from the body and searched for signs of dermal cryptococcosis with fruitless results. The extremities, including the joints, were examined and revealed no lesions. The nervous system also showed no evidence of torulosis, and cerebrospinal fluid, when obtainable, was unclouded and no organisms were grown after culture.

Macroscopic Observations.—Udder.—In some cases, the first impression gained was that of great turgidity and induration of the affected parts. In 1 cow, both fore- and hindquarters of the left side were massively involved and, in another, both quarters on the right side. More than one quarter of an udder was involved in each of the 9 cows examined, but only 1 animal presented disease in all quarters. As the general appearances were similar in several cows with relatively severe infection, one of these might be taken as a descriptive example. In the right hindquarter, whole slices of the udder showed no abnormality until about a third had been cut; the consistency and appearance of normal tissue then rapidly passed into that of a pathological state, which involved the right forequarter in its entirety, front to back and base to teat. The diseased tissue (fig. 1 and 2) was abnormally firm, fleshy, and greyish white. There were occasional haemorrhagic areas. Lobulation was more coarse and very distinct as compared with that of the resilient, normal lactating udder. In some ways, the appearance was reminiscent of the fleshy lobulated induration of human mammary carcinoma. The cut surface was difficult to handle with rubber gloves, for from innumerable areas there oozed a slimy, viscid, greyish white secretion. The teat canals and cisterns of both fore- and hindquarter showed no particular changes. Near the base of the teats, the skin was thickened and was partly adherent to deeper tissues, but there was no apparent break in epidermal continuity. The foregoing appearances are descriptive of all affected quarters of the cows which had been in the more acute stages of infection.

Fig. 2—Cryptococcosis, udder (cow 1799). Whole vertical slice of an affected quarter with similar appearance as in fig. 1 but with compressed remains of normal lactating udder at the upper pole and showing distinct contrast between affected and normal tissues.

Areas of udder which had undergone more involution, and reduction in secretory tissue, were more light grey in color compared with the usual bronze-pink color of glands in the dry state, and numerous small granulomatous foci were present. The secretion material in the ducts (and foci) in these areas was firm and cheesy and did not extrude without pressure, in contrast to the oozing viscid material mentioned previously.

Lymph Nodes.—The gross appearance of the lymph nodes was noteworthy. It should be stressed that the involved nodes were only those which drained the affected sides of the udder. Thus, the corresponding regional supramammary nodes of the affected side were characteristically and constantly involved. In some, the deep inguinal nodes were also involved, but the nodes proximal in the line of drainage to the latter were normal. In the 1 cow with miliary-like foci of cryptococcal infection in the lungs, the deep inguinal nodes (iliac group) on both sides and one of the precrural lymph nodes were enlarged to approximately twice the normal size. None of the other carcase or visceral lymph nodes showed lesions. The increased size of affected supramammary nodes was a most striking feature, and in 1 cow this node was about ten times that of the normal unaffected one on the opposite side. All pathological nodes lay in edematous fascia and fat. When the nodes were cut mesially, they had a waterlogged fleshy appearance, were mottled with confluent greyish white areas and scattered hemorrhages,

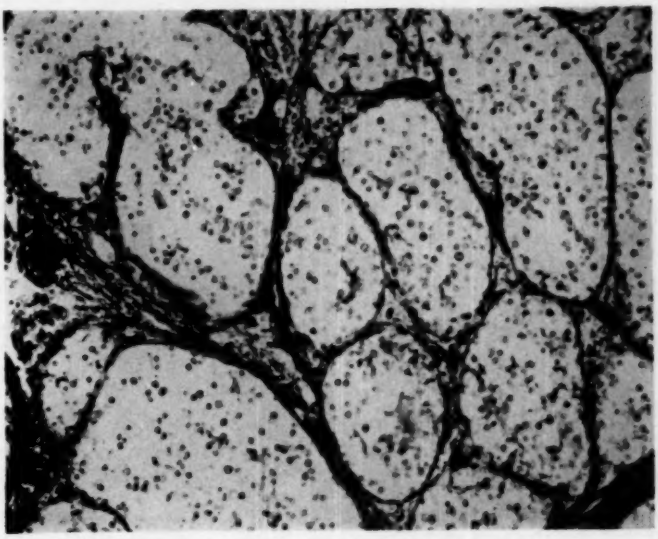

Fig. 3—Cryptococcosis, udder (cow 1799). Showing (a) acute liquefactive type of lesion with complete dissolution of epithelial lining of acini and ductules, (b) lumens filled with organisms, (c) fibrous framework almost intact and absence of inflammatory reaction. Hematoxylin-eosin stain. x 400.

and the cut surface also was slimy. The general appearance was somewhat reminiscent of that seen in bovine leukosis, or in some forms of the noncaseating type of bovine tuberculosis. In some nodes, there were small necrotic, or abscess-like, foci.

Microscopic Observations.—In 1 cow, discrete semicaseous granulomatous lesions with giant cells and epitheloid cells were observed in sections of the skin of the hind legs. It was first thought that these might have been connected with the cryptococcal infection, but Ziehl-Neelsen preparations revealed the presence of slender, curved, acid-fast bacilli. As the herd was reported to be tuberculosis-free, it was concluded that these foci represented those of tuberculoid skin lesions; the cows had not been a reactor to the tuberculin test. The histological difference between these "tuberculoid" lesions and the changes in the udder

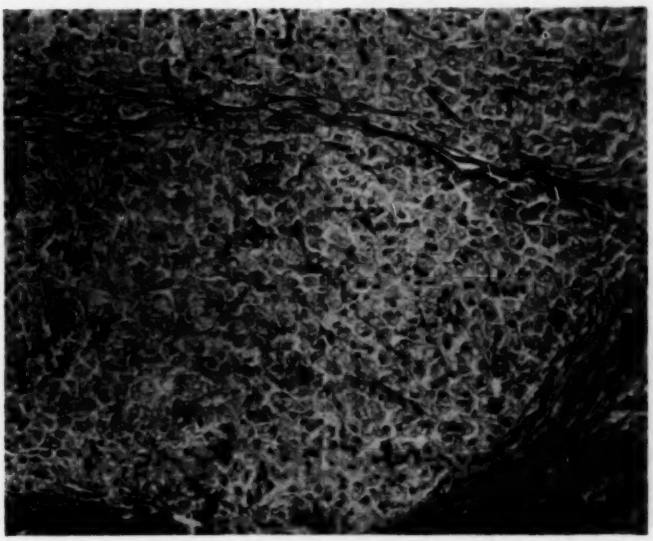

Fig. 4—Cryptococcosis, udder (cow 2385). Showing intense proliferation of large, foamy histiocytes replacing secretory tissue. Many of the cells contain organisms. Hematoxylin-eosin stain. x 400.

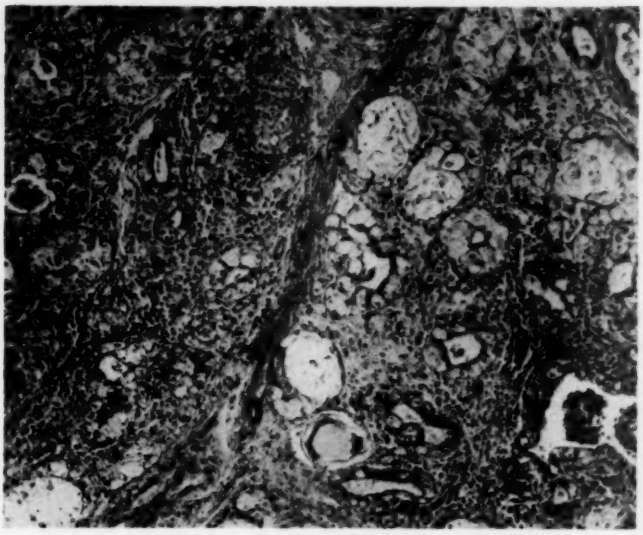

Fig. 5—Cryptococcosis, udder (cow 2385). Another area (same case as fig. 4) showing more chronic type of lesion with some fibrosis and inflammatory reaction; epithelium of ductules and acini lifted up from basement membrane by histiocytes containing cryptococci. Hematoxylin-eosin stain. x 120.

and nodes were so great as to permit of no confusion. Numerous sections of portions of the brain and the spinal cord failed to reveal the presence of meningo-encephalitis.

Udder.—Sections from those quarters which showed no gross macroscopic changes or which were dry revealed little beyond that expected for normal lactating, or "dried-off", udder. In some cases, there were a few, focal, pyogenic granulomas containing Cryptococcus, or there were scattered areas where the involuted udder tissue was diffusely infiltrated with large, foamy histiocytes. These lesions represented the mildest degree of the udder disease which could be recognized histologically.

In the udders which had shown naked-eye evidence of cryptococcal mastitis, only a very thin barrier of fibrous septums separated the normal tissue from pathological areas. As evident from the illustrations (fig. 3, 4, 5),

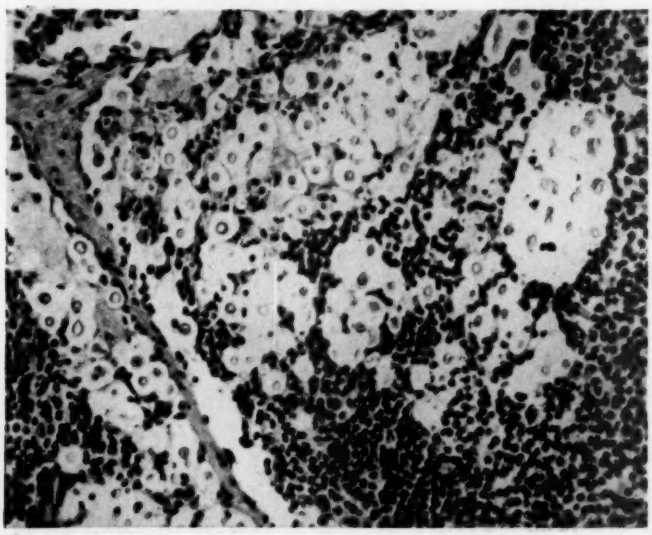

Fig. 6—Cryptococcosis, supramammary lymph node (cow 1799). Showing dilated sinus containing many organisms, with invasion and destruction of cortical lymphoid tissue.

there was considerable variation in the histological picture in different parts of the same affected quarter, and this might have been related to the chronicity of the disease. In the most severe phase (fig. 3), the appearance was interpreted as an intense process of liquefaction affecting all the lining epithelium of the acini, intralobular ductules, and ducts. It seemed as if the lining cells had been almost completely dissolved by the pathogenic action of the Cryptococcus organisms, leaving the skeleton of the basement membrane and interlobular fibrous framework intact (fig. 3). In the glandular structures denuded of epithelium, the organisms were clearly seen in any stained preparation and were innumerable (fig. 3). (The Schiff preparations were advantageously employed for their identification.) The histological appearance of the udder tissue thus conformed with the clinical impression obtained about the nature of the disease, *viz.*, it was a condition characterized by distention and damage of the lining cells of the ducts, ductules, and acini by a massive growth of the yeast cells. Neither the intralobular, nor interlobular, fibrous tissue showed much inflammatory cellular infiltration and, in fact, this was of a surprisingly minor degree. Hemorrhage was not prominent. In some areas, the marked histolytic effect of the yeasts had also affected the fibrous tissue in a similar manner, and had resulted in the formation of large, irregular cysts containing little but the causative organism and presumably, in the fresh state, the mucoid secretion. This picture was similar to the "soapsud" appearance of the cysts in human nervous cases, as illustrated by Globus *et al.*[6]

A variation in the type of histological reaction to severe infection was seen in the udder of 1 cow. This animal, referred to previously, had had infection in all four quarters, and had pulmonary cryptococcal lesions. There was a marked histiocytic reaction in all the affected areas of the udder, and a much less degree of histolytic affect than that described previously. The tissue was massively infiltrated with large, foamy, mononuclear phagocytes, so that in many areas the alveoli and ducts had become completely obliterated by a "tumor-like" infiltration (fig. 4), and numerous Cryptococcus were present in the cytoplasm of these phagocytes. Ducts and ductules, which still remained recognizable, frequently showed parasitized mononuclear phagocytes, singly and in groups, situated between the epithelium and the basement membrane (fig. 5).

In udders with lesions of a more chronic type, there was fibrous tissue proliferation in intra- and interlobular situations, and some foci contained epithelioid cells and thus were more granulomatous in nature.

Small udder abscesses, or pyogenic granulomas, were sometimes encountered with polymorphonuclear, lymphocytic, and histiocytic cells, but giant cells were not found either in the liquefactive or the other types of lesion.

Lymph Nodes.—Histological changes in affected nodes conformed in type to those present in the corresponding udder tissue (fig. 6). In cases with severe involvement of udder, organisms were found scattered throughout the peripheral, cortical, and medullary sinuses, of affected supramammary nodes. Some of the sinuses were greatly dilated and, like the

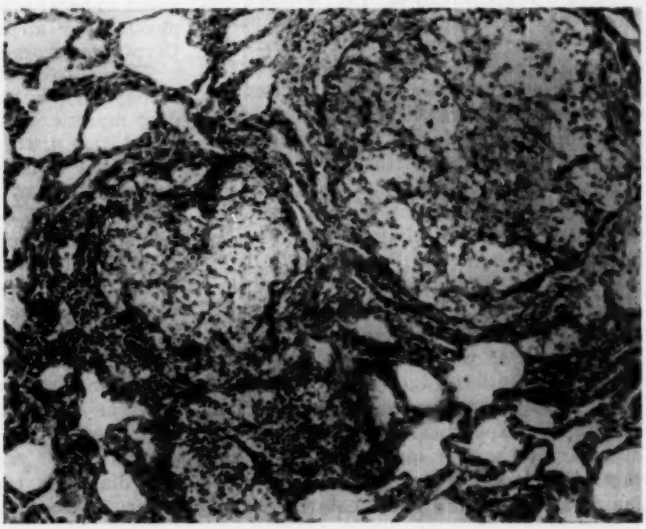

Fig. 7—Cryptococcosis, lung (cow 2385). Showing foci of infection forming small cellular nodules composed mostly of loosely arranged histiocytes and many cryptococci. Hematoxylin-eosin stain. x 120.

udder, contained little but organisms and mucinous secretion. Sinus catarrh was prominent, and some sinuses were thus filled with large, pale-staining macrophages intermixed with numerous organism. Occasionally, there were cysts in some affected nodes, and progressing into a more chronic stage there was extensive infiltration and replacement of the cortical parenchyma with macrophages containing organisms, and necrotic areas merged into abscess formation. The more chronic granulomatous type of lesion seen in the udder was not conspicuous.

Lungs.—The lungs, in one of the 9 cows examined, contained a few focal cryptococcal lesions consisting of small tubercle-like accumulations of foamy macrophages and with many characteristic yeast cells (fig. 7). The lesions did not communicate with bronchi or bronchioles, and might thus have been metastatic foci originating via a hematogenous route from the udder, or by lymphatic paths via the thoracic duct.

DISCUSSION

A study of the pathology of this disease leaves little doubt that cryptococcal mastitis of cows forms a clear-cut disease entity. As in human cryptococcosis, a definite diagnosis of the disease in the cow would require identification of the causative organism, or pathological examination of affected tissues. The *macroscopic* changes in the udder and associated lymph nodes might be indecisive in establishing the precise identity of the infection, but histologically the lesions are indisputably those of a yeast infection. The pathological process, as interpreted by a study of many sections from all the cows, is distinctive, and bears no resemblance to mastitis in cows (or any domestic animal) caused by any other microörganism (*cf.* Little and Plastridge,[7] Nieberle and Cohrs,[8] and Pattison.[9] The reaction to the growth of the yeast in udder tissue is, at first, that of acute epithelial dissolution and liquefaction, becoming more histiocytic or frankly granulomatous in character during later stages of the infection. In that sense, the older name *Torula histolytica* is appropriate even though it has been discarded. We do not know at what extent a concomitant bacterial infection might have complicated the pathological picture in some cases, and manifestly experimental work would be necessary to answer questions of this nature. The lesion in the lymph nodes originates as an intense sinus catarrh following the entry of numerous organisms into the sinuses, which is then succeeded by destruction of lymphoid tissue and by a reactive hyperplasia, and occasionally the formation of abscesses.

There are several significant features in the pathology which are relative to any discussion of mode of infection and pathogenesis, and which indicate that this outbreak of mycotic disease was an example of primary infection of the udder through the teat canal and duct systems. The presence of numerous Cryptococcus organisms in the acini, ductules, and ducts, and the apparent widespread primary involvement of the epithelial lining indicate both rapid growth of the organisms and ramification of infection through intracanalicular channels. In only 1 case were all four quarters affected, and no gross lesions were found in other visceral organs, which would vitiate any suggestion that the udder infection was of hematogenous origin. The spread of the disease to the regional lymph nodes is what might be expected, and the totality of udder infection plus nodal lesions is somewhat analogous to a primary tuberculous mastitis complex. In fact, the gross appearance might have tentatively suggested a possible diagnosis of some forms of bovine tuberculosis.

Although nothing is known of the original source of disease in the first cow infected in the whole outbreak, the pathological data might suggest that all subsequent cows were infected by direct manual, physical, or airborne transmission of the Cryptococcus organisms to the teats.

Torulosis, caused by *C. neoformans* (*T. histolytica*), is one of the most important deep mycotic infections of man, and with a world-wide distribution. The literature reveals that it is being recognized with increasing frequency as the clinico-pathological facts have become better known and, in particular, relating to the involvement of the central nervous system. There are many good recent reviews and original articles (Globus, Gang, and Bergman,[6] Gendel, Ende, and Norman,[5] Cox and Tolhurst,[3] and Binford.[1]) Dermal anl pulmonary forms are clearly distinguished, as well as cases in which the disease is apparently restricted to the central nervous system. Globus *et al.*[6] and others stress that clinical diagnosis of the neurological variety presents a challenging medical problem

and, in fact, is impossible without identification of the causative organism. Little is known about the pathogenesis of the disease in man, and the mode of infection in many cases may be only surmise. It has been stated that no human cases have been proved to derive their infection from animal sources. Yet, as long as the epidemiology is cloaked in obscurity, the possibility of cross-transmission of infection between animals (*e.g.*, cows with mastitis) and man cannot be ignored. The latter is one repercussion which might arise from a consideration of our pathological observations.

SUMMARY

1) The pathology of bovine mastitis caused by *Cryptococcus neoformans* is described.

2) The lesions are characteristic and are distinctive from those of the common types of bacterial bovine mastitis. The process consists primarily of dissolution of the epithelium of the acini, ductules, and ducts caused by massive growth of the organisms in the lumens, followed by a diffuse histiocytic, or by a frankly granulomatous, reaction in the more chronic stages. The regional supramammary lymph nodes were commonly involved. One cow showed foci of cryptococcal infection in the lungs.

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Comparative Immunity Experiments in Cattle Using Live Vaccines Prepared from Agglutinogenic and Nonagglutinogenic Brucella Abortus Strains

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IN THE FIGHT AGAINST bovine brucellosis, there has been unquestionable progress in recent years, much of it in the United States. The use of vaccine made from *Brucella abortus*, strain 19, Bureau of Animal Industry (BAI), discovered in the Unted States, represents at present practically the only means for furthering prophylaxis. This is especially true in certain areas, since the customary measures of identification, isolation, and elimination of carriers do not always receive from specialists or laymen the dedication deserved.

As a consequence of the lack of application of these difficult, complementary, but highly important prophylactic, measures in countries which do not have access to the enormous human and economic potential that the United States has been able to put into the fight against brucellosis, the question has arisen whether all that is done to prevent economic loss (as represented by abortions) produces real benefit for the health of man.

Mingle[1] has stated with great accuracy, "Certainly the human brucellosis cases reported each year can not be disregarded any more than the estimated $100 million annual losses suffered by the livestock industry because of brucellosis in animals." The day will undoubtedly come when a total revision will be imposed on sanitary policies relating to brucellosis, but this will occur only when the specialists can provide a simpler and more effective method of combat. This goal will be reached as soon as new roads are opened to research and when analysis of this problem is approached from different angles, an objective practically unreachable now, considering the relatively unilateral way this campaign is furthered.

There are many unexplained factors happening in vaccinated lots, as well as in the course and evolution of infection in its different degrees in the same. Frequently, there is talk of antiabortive or anti-infectious immunity in bovine brucellosis. If there is immunity, it has to be anti-infectious. Infectious abortion is not a disease but a manifestation or consequence of brucellosis, and the process that determines the reduction of abortions in natural immunity is directly dependent on the degree of anti-infectious resistance, as is demonstrated by the animals naturally resistant to the infection which do not abort.

It is also true that in nature infected animals acquire, after one or two abortions, a marked resistance towards a repetition of the phenomenon. A similar result is obtained in great numbers of vaccinated animals which, even though infected, never abort. It appears quite unorthodox to define immunity as a mechanism which manifests itself functionally only in a determined organ, while it allows the microörganisms to multiply, passing ultimately to the exterior to invade other hosts.

The successful experiment performed by Cotton, Buck, and Smith[2] in 1934 proved to be an important addition to the study of prevention of bovine brucellosis, even though the results obtained were not reproduced in similar conditions by other investigators. In that experiment, the antiabortive results were perfect in the group of animals vaccinated with strain 19. From the anti-infectious standpoint, only 1 of the 9 animals became infected, as judged by isolation of the organism from the colostrum, while from 10 of 16 controls *Br. abortus* was isolated from the uterus and colostrum. In that publication, there was an omission of data pertaining to the postpartum agglutination response of these animals. This observation is of importance since it is well known that a considerable number of infected animals can give birth to normal calves and, even though they may not eliminate the organism through the udder or uterine exudates, they reveal their infected state by an increase in the agglutination titer between the sixth and tenth day after parturition.

Haring and Traum[3] in 1941 reported a series of investigations for the appraisal of the

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immunizing properties of strain 19 in calves. They mention that with moderate doses of virulent organisms they obtained as much as 100 per cent protection, while with doses of several thousand million organisms some of the animals developed the disease and aborted. These authors do not say whether these 100 per cent protection figures referred to abortion values or if it also included anti-infectious resistance. These investigators consider that the best immunity test is natural field exposure, with a great number of vaccinated animals and numerous controls. In trials performed under these conditions on almost 10,000 animals, there were 94 per cent normal births in the vaccinated animals against 86.1 per cent in the controls. No data are given on the incidence of infection in the vaccinated animals in comparison to the controls.

Birch, Gilman, and Stone[4] (1945) used natural exposure and the vaccinated animals gave 94.27 per cent normal births in comparison to the controls, which gave 76.92 per cent. The anti-infectious immunity was not as good, because even though the infection caused abortion in the controls in a proportion 13.33 per cent higher than the vaccinated group, a high number of these (53.34%) became infected, with elimination of the organism from the udder or uterus as well as with positive agglutination titers of 1:100 or above.

Another immunity test was performed by artificial exposure at the third and fourth pregnancies on animals vaccinated as calves and heifers by Beach, Irwin, and Berman[5] in 1947. These investigators used an infective dose of 30 to 35 million organisms by conjunctival exposure which, considering the results obtained, was said to be too severe, even though they recognize that in a previous experience they used a much higher infective dose. Of 29 animals vaccinated and infected, 12 had normal births, 4 gave birth to weak calves, and 13 aborted. As such, the anti-abortive values reached practically 50 per cent protection, whereas the anti-infectious values were very low, since 25 of the 29 animals (86.2%) eliminated the organisms or possessed agglutination titers of 1: 100 or higher. All the controls aborted and all were infected.

We sustain the thesis that if the control of brucellosis has to be based on the use of vaccines, these must be nonagglutinogenic and will have to confer the highest anti-infectious protection values possible, in order that:

a) its use can be extended with advantage and without limitations to all livestock raising countries;

b) prophylactic work may crystalize into a definite objective: the raising of an effective barrier which may prevent progress of the infection. This would be of benefit to human health and also to livestock, without interfering in the detection of outbreaks of the disease which may require urgent treatment by more drastic methods.

A series of anti-Brucella bovine immunity experiments have been in progress since 1947 in order to test the anti-infectious and anti-abortive capacity of vaccines prepared from different strains of *Br. abortus*, using strain 19 (BAI) as a standard of comparison. This report represents a series of observations of a preliminary nature. We are at present repeating these immunity tests on a larger scale (200 animals) and hope that the errors incurred in the present experiment may serve to prevent these difficulties in the future.

EXPERIMENTAL METHODS

Among the strains of Brucella studied were the "intermediate type," nonagglutinogenic strains B and BM which were discussed in a previous paper by Rosenbusch[6] summarizing the results of their field application on 1,250,000 animals through 1920 to 1946. The other two strains studied in this comparative test were

TABLE I—Orientation Anti-Brucella Immunity Test Strains 19, B, and BM Vaccines

N° OF ANIMAL	Agglut. 25/2	VACCINATION 17/3/47	AGGLUTINATION REACTIONS 8/4	23/5	14/6	25/7	4/9	27/9	13/10	OBSERVATIONS
8		St. "B"		C			NB			NORMAL BIRTH 14/9
2		St. "BM" with Al. Hid.		C		NB				NORMAL BIRTH 10/8
6		St. "BM" with Al. Hid.		C		NB				NORMAL BIRTH 12/8
3		St. "19"		C		WC				WEAK CALF 27/8
7		St. "19"		C		NB				NORMAL BIRTH 3/7
14		St. "BM" without Al. Hid.		C			AB			Stillbirth (Ab. Infec.) 13/9
15		St. "BM" without Al. Hid.		C		WC				WEAK CALF 28/7

C - CONJUNCTIVAL INFECTION
NB - NORMAL BIRTH
AB - ABORTION
WC - WEAK CALF
Al. Hid. - ALUMINIUM HIDROXIDE

■ - POSITIVE AGGLUT. 1/100 OR MORE
▩ - POSITIVE AGGLUT. 1/50
▨ - POSITIVE AGGLUT. 1/25
□ - NEGATIVE AGGLUT.

strain 19 (BAI) and one avirulent agglutinogenic intermediate strain 37 of our laboratory's collection, which has been used since 1920 for the preparation of antigen for the agglutination test. This strain was selected on the basis of its high and stable antigenic nature, considering that it might also be highly immunogenic through a probable interrelation of both properties.

The vaccines used in these tests were freshly prepared. Strain 19 vaccine was prepared from a strain recently provided by the Argentine Department of Agriculture. It was grown and maintained under the conditions recommended.

Strain 37 was grown in the same way as strain 19, but it was maintained in passage on a serumized meat-infusion agar. Strain B was grown and maintained on the same meat-infusion agar with 10 per cent serum. These vaccines were used in the following doses: strain 19, 5 cc. of a bacterial suspension containing 12 billion live organisms per cubic centimeter; strain 37 in a 10-cc. dose of 12 billion live organisms per cubic centimeter; strain B in a 5-cc. dose of 12 to 14 billion live organisms per cubic centimeter.

The animals on both tests were kept together and under natural conditions throughout the length of the test. These animals originated from dairy herds and were of the Shorthorn or Argentine Holstein-Friesian breeds. The majority of the animals used in these tests were vaccinated as heifers, with ages ranging from 10 to 18 months. A few were vaccinated at 2 years of age. All were tested for infection by the agglutination test prior to vaccination and at approximately monthly intervals thereafter. All the animals were negative prior to vaccination.

Exposure was performed in the two experiments with different virulent strains. A recently isolated virulent field strain was used in the first (orientation) experiment, using 3 million live organisms conjunctivally.

For the second and main experiment, the virulent strain (strain RT) used was selected on the basis of its greater guinea pig infectivity and its stability in virulence. In the guinea pig, this strain had proved to possess a minimum infective dose of 150 to 200 organisms. This strain was kept at this level of virulence by guinea pig passage and lyophilization.

Infection in this experiment was performed conjunctivally, subcutaneously, or, in some cases, intradermally. The conjunctival and intradermal dose was of 1,800,000 organisms while the subcutaneous dose was 3,600,000.

The majority of the animals of the second experiment (67%) were exposed to infection while nonpregnant. The remainder showed pregnancies of two to six months.

Infection in most animals was established by the study of the agglutinin titers present at monthly intervals. A titer of 1 : 100 or above, maintained through repeated observations, was the criterion for an infected case. The presence of infection was further demonstrated in the orientation test and in those performed on pregnant animals by the isolation of the infective organism from the tissues of guinea pigs inoculated with fetal membranes and the colostrum from normal births or cases of abortion. This method of analysis of infection in the present test paralleled perfectly the results of the agglutination test, but it detected less cases of infection than the latter, as seen in tables 2 and 3.

RESULTS

Preliminary Experiment (See Table 1).—This test was performed on 12 animals in 1947, with the purpose of providing an orientation in the bovine immunity values produced by several vaccines prepared with the nonagglutinogenic strains B and BM in comparison with strain 19 (BAI).

At this time, 2 animals were vaccinated with strain 19, 1 with the nonagglutinogenic strain B, 2 with the nonagglutinogenic strain BM, and 2 with strain BM with 10 per cent aluminum hydroxide. In addition, 2 animals were left as controls but they were too far along in pregnancy and they gave birth to calves before or immediately after exposure so that they were lost for the purpose of the test. The same happened with 2 additional animals vaccinated with strain B and 1 with strain BM.

All the animals of this test were vaccinated in March, 1947, with pregnancies varying between three and eight months. Both animals vaccinated with strain 19 developed agglutinins after vaccination, with titers of 1 : 50 and 1 : 100, while of those vaccinated with strains B and BM with aluminum hydroxide only 1 produced agglutination titers up to 1 : 25.

Two months later, the animals were exposed by the conjunctival route. The animals vaccinated with strain B and strain BM with aluminum hydroxide demonstrated a perfect antiabortive resistance. All 3 calved normally. Only 1 presented agglutinins up to 1 : 50 after exposure.

Both animals vaccinated with strain 19 showed positive agglutination reactions of 1 : 50 to 1 : 100 at irregular intervals after exposure. One of these animals gave birth to a weak calf three months after being exposed, while it showed a considerable increase in agglutination titers (with readings higher than 1 : 100 after calving), a probable indication of infection.

TABLE 2—Anti-Infectious Brucella Immunity Test Strain 37 Vaccine (Pregnant Animals)

N.	1949 3	4	5	6	7	8	9	10	11	12	1950 1	2	3	4	5	6	7	8	9	10	11	12	1951 1	2	NB or AB	OBSERVAT.	GUINEA PIG INOCULAT.
04	V										C								NB						NB 5/9		NEGATIVE
091	V										C								NB						NB 26/9		
395	V										C									NB					NB 2/10	DYSTOCIA	NEGATIVE
405	V										C							NB							NB 27/8		
526	V										C								NB						NB 14/9		NEGATIVE
708	V										C								NB						NB 18/9		NEGATIVE
727	V										C									NB					NB 10/10		NEGATIVE
138	V										S								NB						NB 5/9	RET. PLAC.	NEGATIVE
216	V										S						AB								AB 12/7	7 month fetus	POSITIVE
234	V										S								NB						NB 22/9		NEGATIVE
286	V										S								NB						NB 8/9	Pus in cotyledons	NEGATIVE
397	V										S							NB							NB 27/8		
403	V										S						NB								NB 25/7	CALF DIED	NEGATIVE
421	V										S							AB							AB 3/9	STILLBIRTH	
598	V										S								NB						NB 26/9		NEGATIVE
317	V										I							AB							AB 9/8		POSITIVE
383	V										I								NB						NB 8/9		NEGATIVE
408	V										I										NB				NB 22/11		NEGATIVE
815	V										I								NB						NB 5/9		NEGATIVE

V - VACCINATED
C - CONJUNCTIVAL INFECTION
S - SUBCUTANEOUS INFECTION
I - INTRADERMIC INFECTION
NB - NORMAL BIRTH
AB - ABORTION
■ - POSITIVE AGGLUT. 1/100 OR MORE
▩ - POSITIVE AGGLUT. 1/50
▨ - POSITIVE AGGLUT. 1/25
□ - NEGATIVE AGGLUTINATION

TABLE 3—Anti--infectious Brucella Immunity Test Nonvaccinated Controls (Pregnant Animals)

N.	1949 3	4	5	6	7	8	9	10	11	12	1950 1	2	3	4	5	6	7	8	9	10	11	12	1951 1	2	NB or AB	OBSERVAT.	GUINEA PIG INOCULAT.
028											C									AB					AB 1/10	STILLBIRTH	NEGATIVE
033											C									NB					NB 1/10		NEGATIVE
035											C									NB					NB 25/10		NEGATIVE
932											C								NB						NB 10/9		NEGATIVE
981											C							NB							NB 27/8		NEGATIVE
031											S								NB						NB 10/9		NEGATIVE
772											S			AB											AB 14/4	6 MONTH FETUS	NEGATIVE
808											S							AB							AB 28/8		POSITIVE
812											S								NB						NB 14/9		
858											S									NB					NB 2/10		NEGATIVE
986											S							AB							AB 8/8	LARGE FETUS	NEGATIVE

V - VACCINATED
C - CONJUNCTIVAL INFECTION
S - SUBCUTANEOUS INFECTION
NB - NORMAL BIRTH
AB - ABORTION
■ POSITIVE AGGLUT. 1/100 OR MORE
▩ POSITIVE AGGLUT. 1/50
▨ POSITIVE AGGLUT. 1/25
□ NEGATIVE AGGLUTINATION

Both animals vaccinated with strain BM without aluminum hydroxide became infected. One aborted four months after exposure, with isolation of organisms from the fetus, while the other gave birth to a weak calf. Both animals presented high agglutination titers before and after calving or aborting.

It is unfortunate that we were unable to have adequate controls in this test, but the figures of infection shown by the animals vaccinated with strain BM without aluminum hydroxide serve us as a good evidence of the degree of exposure to which this lot has been subjected.

Comparative Bovine Immunity Experiment.—A new series of immunity tests was started in 1949 with 89 animals. At this time, 48 heifers were vaccinated with our agglutinogenic strain 37, 15 with the non-agglutinogenic strain B, and 15 others with a vaccine prepared from strain 19 (BAI), while 11 heifers of the same age were left unvaccinated as controls. The vaccination age of these animals varied between 10 and 18 months.

Agglutination tests were performed on all these animals prior to vaccination. All proved to be negative. Twenty-five days after vaccination with strain 37, 85 per cent of the animals presented agglutination values of 1 : 50 to 1 :100 or more. However, these disappeared in the majority of the animals within one or two months.

The group of animals vaccinated with strain 19 showed an apparently irregular reaction determined by the appearance of agglutination titers of 1 : 25 to 1 : 50 in only 33 per cent of the animals. These results may be due to the fact that too much time was allowed to elapse (seventy-one days) between the vaccination date and the first agglutination test following (Dec. 15, 1949, and Feb. 24, 1950). We assume that there could have been a loss in antibodies within this first period in the group that did not show agglutinins. There are references in the literature relating to the fact that there is normally a pronounced fall in the agglutination titers produced by strain 19 vaccine between the second and the third months, with values higher than 44 per cent (Hubbard[7] and Beach, Irwin, and Berman[5]).

None of the animals vaccinated with strain B showed agglutinins as the result of vaccination.

Artificial exposure was performed simultaneously in all the lots of animals of this experiment in February, 1950, two to ten months after vaccination, using the conjunctival, subcutaneous, or intradermal route of infection.

A great number of the animals in this experiment were exposed to infection while nonpregnant. However, 19 heifers of the lot vaccinated with strain 37, and all the controls, showed pregnancies varying between two and six months at the time of infection.

Pregnant Lot (See Tables 2 and 3).—Within the group of 7 animals vaccinated with strain 37, which were exposed conjunctivally ten months after vaccination, infection occurred in 28 per cent, judging by the agglutination test, whereas there were no abortions (table 2). The infection value in the unvaccinated control group of 5 animals (table 3) was 60 per cent whereas the abortion figure was 20 per cent. The case of abortion mentioned was a stillbirth and infection was not verified by culture isolation from the guinea pig injected with fetal membranes nor by the agglutination test.

All the positive agglutination reactions in the vaccinated and control groups appeared after calving. In no case was it possible to isolate the infective organism from the tissues of the guinea pigs inoculated with the fetal membranes or colostrum of any of these animals.

In conclusion, we have to admit that exposure in this experiment was carried out somewhat too late, considering the degree of pregnancy of the animals and the fact that it was performed conjunctivally, a method of infection which is apparently slower in its effects.

Within the group of 12 animals exposed subcutaneously or intradermally, the animals vaccinated with strain 37 presented infection figures of 41 per cent, while those of the 6 unvaccinated controls presented 17 per cent, judging infection only from the standpoint of a positive agglutination test. The cultures and guinea pig inoculations performed with fetal membranes and colostrum samples from normal births, as well as from abortions, demonstrated only 2 cases of infection in the vaccinated group (16%). The abortion rate was 25 per cent for the vaccinated lot and 50 per cent for the controls. Infection manifested itself

in this group from the first to the sixth month of exposure.

Nonpregnant Lot (See Tables 4, 5 and 6).—This lot includes three separate vaccine groups. Unfortuntely, it was not possible to include a nonvaccinated group. Vaccine was applied in these cases in young unserved heifers.

Artificial exposure was performed by the conjunctival or subcutaneous route two to ten months later. The results obtained in these experiments follow.

Strain 37 (See Table 4).—The lot of 16 animals exposed conjunctivally ten months after vaccination gave infection figures of 38 per cent, judged on the basis of the agglutination test, and 18 per cent aborted. There were also 7 normal births, 3 animals died or were discarded, 2 were recently pregnant, and 1 never became pregnant.

Of the lot of 13 animals exposed subcutaneously or intradermally, there was only 1 case of infection which manifested itself by an agglutination reaction of 1 : 100

TABLE 4—Anti-infectious Brucella Immunity Test Strain 37 Vaccine (Nonpregnant Animals)

N.	1949												1950												1951									NB or AB	OBSERVAT.
	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9		
03		V									C										d													
50		V									C																	NB					NB 10/5	
219		V									C																	NB					NB 10/5	
236		V									C																		NB				NB 6/7	
303		V									C										d													
320		V									C																							UNOBSERV. ABORT.
374		V									C																		NB				NB 12/7	
379		V									C																		NB				NB 10/8	
382		V									C																							REC. PREGNANCY
387		V									C																							REC. PREGNANCY
449		V									C																							NOT. PREGNANT
468		V									C																							UNOBSERV. ABORT.
512		V									C																		NB				NB 28/7	
548		V									C																NB						NB 15/5	
582		V									C		D																					
883		V									C																							STILLBIRTH 15/10
4		V									S																	NB					NB 7/7	
076		V									S																		D					
278		V									S																	NB					NB 5/6	
410		V									S																		D					
419		V									S																					NB		NB 20/9	
445		V									S																							NB 28/10	
482		V									S										d													
564		V									S																	d						
586		V									S																NB						NB 5/15	
599		V									S																	NB					NB 28/6	
693		V									S																	NB					NB 1/7	
287		V									I																						NB 1/11	
506		V									I																	NB					NB 30/6	

V - VACCINATED

C - CONJUNCTIVAL INFECTION

S - SUBCUTANEOUS INFECTION

I - INTRADERMIC INFECTION

NB - NORMAL BIRTH

■ - POSITIVE AGGLUT. 1/100 OR MORE

▩ - POSITIVE AGGLUT. 1/50

▨ - POSITIVE AGGLUT. 1/25

□ - NEGATIVE AGGLUT.

d - DISCARDED

D - DIED

for a period of two months, eight months after infection, after which it gradually disappeared. In this lot there were no abortions. There were 9 normal births while 4 animals died or were discarded.

Strain B (See Table 5).—The group of 7 animals exposed by the conjunctival route two months after vaccination did not present any case of infection or of abortion. One animal of this group, six months after exposure, developed an agglutination titer of 1 : 50 which decreased in the next month to 1 : 25, then disappeared and reappeared alternately during the following 18 months. In this group, there were 5 normal births, 1 animal died, and 1 presented a recent pregnancy.

In the lot of 8 animals exposed subcutaneously, there were 2 which presented agglutinin titers of 1 : 50 during three to four months after infection, after which there was a reduction to values of 1 : 25 or negative. Another group of 3 animals showed agglutinin titers of 1 : 25 for short periods. In this lot, there were 7 normal births and 1 animal was discarded.

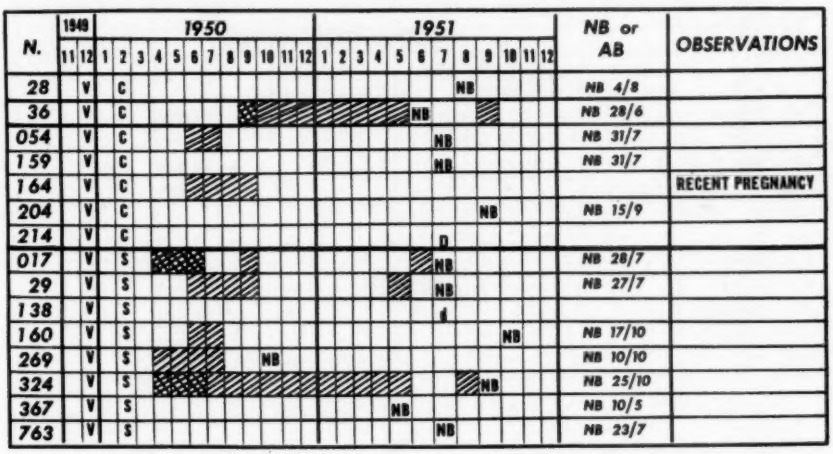
Concluding, we can state that neither conjunctival nor subcutaneous exposure was able to affect the anti-infectious immunity produced by this strain. It can only be said that subcutaneous exposure has produced a slight and fading rise in the agglutination titer of some animals.

Strain 19 (See Table 6).—From the lot of 5 animals which presented positive agglutination reactions seventy-one days after vaccination and which lost them afterward in a total or partial way in the next four months, the following results were obtained.

By the conjunctival exposure 2 of 3 animals (66%) were infected seven or eight months later, without the production of abortions; by subcutaneous exposure, there were no noticeable changes in agglutination reactions, with values ranging between 1 : 25 and 1 : 50 during prolonged periods after infection, a reaction which might also be due to the vaccine. In this group there were no abortions, there were 4 normal births, while 1 animal was in advanced pregnancy at the end of the test.

Of the group of 10 animals which did not present agglutinins on the seventy-first day after vaccination, the following results were obtained. By conjunctival exposure there were no infected animals nor were there any cases of abortion; by subcutaneous exposure, there was 1 infected animal

TABLE 5—Anti-infectious Brucella Immunity Test Strain B Vaccine (Nonpregnant Animals)



V - VACCINATED
C - CONJUNCTIVAL INFECTION
S - SUBCUTANEOUS INFECTION
NB - NORMAL BIRTH

NEGATIVE AGGLUT.
POSITIVE AGGLUT. 1/50
POSITIVE AGGLUT. 1/25
d - DISCARDED
D - DIED

(17%), but there were no visible abortions. It is of interest to note that the infected animal proved to be sterile, probably as a result of the same infection. In this group, there were 7 normal pregnancies and 3 animals that did not become pregnant.

DISCUSSION

An observation of great interest was made from the study of this preliminary experiment on the comparative immunological value of different *Br. abortus* strains in relation to the varying forms of artificial exposure. The animals of all tests which did not produce agglutinins, either as a result of the type of organism used in the vaccine (strain B), or which possibly lost this reaction rapidly after vaccination (strain 19), had the best anti-infectious as as well as antiabortive immunity.

The relation between the processes of agglutinin production and immunity has been treated repeatedly in the literature. Beach, Irwin, and Berman[5] stated that there was no relation between the postvaccinal agglutination peak and the subsequent resistance of the animal. In this sense, Hubbard[7] reached the same conclusion on the relationship between agglutinins and immunity in his analysis of the factors intervening in calfhood vaccination with strain 19.

Furthermore, Carcamo, and DeDiego[8] stated that strain 19 vaccine, killed by heat or by other agents, produced a strong agglutinin response in the vaccinated animals which did not differ from that produced by the same strain in a living state. It is well known that strain 19 is not effective as an immunizing agent when applied as a bacterin.

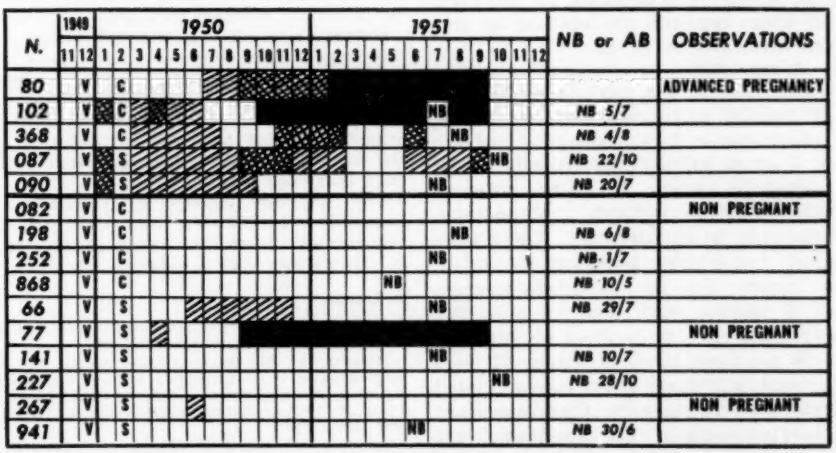
Live, Sperling, and Stubbs,[9] using guinea pigs as the test animal, demonstrated by the use of ultrasonic filtrates that immunity is not necessarily related to the production of agglutinins.

The work of McEwen[10] with his "rough" strain 45-20 which does not produce agglutinins, while supposedly producing appreciable values of immunity in cattle, is additional evidence of the lack of relation between immunity and agglutinin production.

Rosenbusch[6] in 1947 reported on the large-scale field study, for more than a decade, on the vaccination of over a million animals using the intermediate type nonagglutinogenic MBB *Br. abortus* strains. In this report, only antiabortive observations were taken into account.

The analysis of anti-Brucella immunity, strictly on a basis of its antiabortive effects, resolves only the economic aspects of the problem. In our opinion, this objective is only secondary. Our aim is to develop a vaccine which may produce an adequate anti-infectious immunity, thereby eliminating abortions and also the numerous secondary problems related to this disease.

TABLE 6—Anti-infectious Brucella Immunity Test Strain 19 Vaccine (Nonpregnant Animals)


N.
1949
1950
1951
11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12
NB or AB
OBSERVATIONS
80 V C ADVANCED PREGNANCY
102 V C NB NB 5/7
368 V C NB NB 4/8
087 V S NB NB 22/10
090 V S NB NB 20/7
082 V C NON PREGNANT
198 V C NB NB 6/8
252 V C NB NB 1/7
868 V C NB NB 10/5
66 V S NB NB 29/7
77 V S NON PREGNANT
141 V S NB NB 10/7
227 V S NB NB 28/10
267 V S NON PREGNANT
941 V S NB NB 30/6
V - VACCINATED
C - CONJUNCTIVAL INFECTION
S - SUBCUTANEOUS INFECTION
NB - NORMAL BIRTH
POSITIVE AGGLUT. 1/100 OR MORE
POSITIVE AGGLUT. 1/50
POSITIVE AGGLUT. 1/25
NEGATIVE AGGLUTINATION

Bovine brucellosis is not simply an animal disease and it can not be treated separately from the problems involving human health. The protection of human health has become necessary as a consequence of the studies made which denote a great prevalence of this disease in man.

We can expect only limited benefits for human health if anti-Brucella vaccination serves only to protect the cow from the loss of its offspring while it provides only a limited protection against infection. Considering the human health aspects of the problem, immunity should be studied mainly from the standpoint of reducing or preventing the advance of infection.

This objective, difficult to reach with the vaccines presently in use, has imposed the application of various prophylactic measures, including the elimination of carriers. This step, however, is of difficult execution, partly because of the relatively poor immunogenicity of the present vaccine strains, as well as by their interference in the control program through their stimulation of blood antibodies which are indistinguishable from those of disease.

SUMMARY AND CONCLUSIONS

1) An orientation test performed on 7 animals in 1947 showed that two intermediate nonagglutinogenic strains produced a better immunity than strain 19; however, one of these required the addition of aluminum hydroxide to do so.

2) A new series of comparative immunity experiments was performed in 1949 on 89 heifers vaccinated with strain 19, the intermediate type, nonagglutinogenic strain B or an avirulent intermediate type agglutinogenic strain 37, using different artificial exposure methods (conjunctival, subcutaneous or intradermal). The results obtained demonstrate superior anti-infectious values for the nonagglutinogenic strain B vaccine, with total infection figures of 0 per cent, while those of strain 19 were 20 per cent, those of strain 37 were 25 per cent, and those of the nonvaccinated controls were 36 per cent. Complementary antiabortive values of this experiment were 0 per cent abortions for strain B; 0 per cent for strain 19; 12 per cent for strain 37, and 10 per cent for the nonvaccinated controls.

3) Classifying the immunity results obtained according to routes of exposure and whether the animals had been served or not, there is a much quicker and more severe degree of infection in the pregnant lot with the subcutaneous exposure, whereas this type of infection seemed to act as a hyperimmunizing factor without the production of agglutinins in the nonpregnant lot. Conjunctival infection appeared to be most natural in its effects, producing the highest percentage of infection in most lots. Infection under these conditions manifested itself only after six months of exposure.

4) It is noteworthy that the best anti-infectious immunity was obtained in the animals which did not show agglutinins after vaccination, due to strain characteristics or to reasons of possible early disappearance of agglutinins (after seventy days). By coincidence, the same anti-infectious results were obtained in a group of 5 animals vaccinated with the agglutinogenic strain 37, which did not show agglutinins after vaccination.

Editor's Note.—This manuscript is being published because it is the result of independent research in a foreign environment and because it claims some desirable achievements. Apparently, some of the findings are at variance with those of several other research workers, but they seem to have been honestly arrived at and may be explained. This work might stimulate a re-study of some phases of our generally accepted concepts.

Reviewers of this manuscript believe that the experiments recorded might have been better controlled and that the unusual response following the use of strain 19 vaccine could mean either that the vaccine had been improperly handled and was nonviable or that the method of testing for agglutinins differs from that used by others.

The *American Journal of Veterinary Research* therefore invites comments regarding this manuscript, so that any false impressions it might create may be pointed out.

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The Activity of Streptococcus Agalactiae in Milk Possibly Influenced by the Ration

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A SEARCH was made for factors that through their effect on dairy cows might influence the milk produced by the animals as regards its suitability as a medium for *Streptococcus agalactiae*. Should such factors exist, they conceivably might influence disease conditions of udders associated with this infection. Their existence would not be incompatible with some of the suggested explanations concerning the means by which the pathological conditions are brought about, including those of hypersensitivity of the udder tissues to the presence of *Str. agalactiae*[10] and the production of irritant products from the milk.[6, 8]

Variations between herds as to the suitability of their milk for acid production by cheese starters has been noted.[11] Between cows differences exist in the bacteriostatic and bacteriocidal properties of their milk.[8] These properties of milk were considered by Murphy[5] to be basic factors in the resistance of udders to infection. The inhibitory action for *Salmonella typhosa* of the milk from a cow while on pasture was observed to be greater than when she was receiving ensilage and grain.[1] Others[4] failed to note any such variation associated with the feed when using *Streptococcus pyogenes* as the test organism.

A method whereby variation in activity of *Str. agalctiae* in milk samples could be measured was evolved in preliminary work. It consisted of inoculating the samples either with broth cultures or with milk from an infected cow and, following incubation, measuring the quantity of sodium hydroxide required to neutralize the acid formed. Among the factors given limited consideration as possibly capable of influencing the milk as a medium for the bacteria were the feeds eaten by the animals, the feeding of vitamin A, of citrates and phosphates, the intravenous injection of the latter, of vitamin E, and of ascorbic acid.

Only in the case of the quality and quantity of roughage being eaten by the cows was evidence obtained indicating possible involvment of any of these factors to an appreciable degree. For instance, samples from 6 cows were collected and inoculated in September and the process repeated at intervals until the end of February for a total of 24 times. The quantities of sodium hydroxide required to neutralize the acid formed tended on the average to be lowest during periods when the animals were receiving the better qualities of hay.

Milk samples from a small herd of 8 cows were examined five times in a similar manner during the same period. The animals were on pasture during the first two months and received very little grain. As the pasture became poor, grain was increased and corn stover added to the ration. For the last two months, the ration consisted of timothy hay of variable quality and grain. The average quantities of sodium hydroxide required to neutralize the acid produced in the inoculated samples at each successive test increased until it was several times greater than at the beginning of the period of observation. As would be expected, all results were not in agreement with the above. For instance, the feed of 3 cows in advanced lactation was changed for three weeks from a ration of alfalfa hay, silage, and grain to one of but straw and grain. Variations that appeared to be related to the change in diet were not produced in the quantities of sodium hydroxide required to neutralize the acid in their samples.

These experiences stimulated the initiating of another experiment for the purpose of obtaining further information regarding the possible influence of the diet on the milk as a medium for *Str. agalactiae*. This was considered as offering a partial substitute for experiments involving exposure of animals to disease organisms, an approach which was not possible to follow in the herds available for study.

EXPERIMENTAL

Animals and Feed.—Sixteen cows were used in this experiment and these were divided into two groups, each composed of 4 Holstein-Friesians and 4 Jerseys. The animals were paired off as closely as possible on the basis of breed, quantity of milk being produced, stage of lactation, and age. Four Jersey cows, 2 in each group, were dry at the time the experiment was started and milk samples from them were not available until the ninth week. However, from the beginning of the experiment they received the same feeds as the other cows in

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their respective groups. One group was fed a ration of alfalfa hay (free choice) along with silage, consisting primarily of meadow crops, and a simple 14.5 per cent protein grain mixture consisting of corn, oats, bran, soybean oil meal, and salt. The other group received grass hay (free choice) made for the most part from timothy, and a grain mixture composed of the same feeds but containing 18.5 per cent protein. All cows had received quite similar rations prior to the start of the experiment. Alfalfa hay had been fed, generally of medium quality, along with silage and the simple grain mixture.

The change-over to the specified feeding programs was accomplished in a gradual manner during January, so that by the first week in February all animals had been fed for some days according to schedule. It was possible to carry on the experiment until April 19. During February, March, and April, the average consumption of feeds per day for the 6-cow group receiving alfalfa hay was approximately 15 lb. of alfalfa hay, 52 lb. of silage, and 9.5 lb. of grain. The other group of 6 cows consumed an average per day of approximately 14.3 lb. of timothy hay, and 18.0 lb. of grain. The average milk production per day for the group receiving alfalfa hay and silage was 32.7 lb. for the first week of February and 27.0 lb. during the last week of the experiment. This group averaged 30.4 lb. per day (range 20.5 to 50.8 lb.) for the period covering February, March, and part of April. The average production for the 6 cows in the timothy hay fed group was 27.5 lb. per day (range 21.4 to 33.7 lb.) for the same period. The average for the first week of February was 35.7 lb. per day and 23.3 lb. per day during the last week of the experiment.

An effort was made to pick the best quality alfalfa hay available to feed to the group assigned to this type of ration. That fed during March was among the best obtained during the experiment. Prior to and after this period, much of the hay was of secondary quality. Almost all the grass hay was of rather poor quality. Analysis indicated that it contained 2.9 μg. per gram of carotene and 4.5 per cent protein. An exception to this was the hay fed to this group of cows on April 1 and 2 when it was of better quality and contained some clover. Analysis then showed the presence of 8.8 μg. per gram of carotene and 8.9 per cent protein.

Acid Production by Streptococcus Agalactiae in Milk Samples.—Quarter milk samples were collected aseptically at weekly intervals, being obtained three to four hours after usual morning milking. Duplicate 10-ml. Hotis tests[9] were set up and 1 of each pair was inoculated with a culture of *Str. agalactiae*. This was followed by incubation at 37 C. for twenty-four hours. The culture was carried from week to week on beef extract liver infusion blood agar. It was inoculated from the agar into dextrose broth and after 24-hour incubation at 37 C. another tube was inoculated from the first. Twenty-four hours later this latter broth culture was used to inoculate the Hotis test. A straight platinum wire needle was used to accomplish this inoculation. These procedures, which were arrived at by trial and error, resulted in sufficiently limited reactions in the milk samples to permit differences to be detected between samples in suitability as media for *Str. agalactiae*. Following incubation of the Hotis tests for twenty-four hours at 37 C., the quantity of 0.1 N sodium hydroxide solution was determined which was required to neutralize the acid and return the samples to approximately their original color. The duplicate tubes provided some assistance in deciding the color back to which inoculated samples from uninfected animals should be titrated. They provided also some indication as to whether the samples were already infected with *Str. agalactiae*.

The average quantities of 0.1 N sodium hydroxide solution required to neutralize the acid formed in the inoculated milk samples from the two groups of cows are presented graphically (fig. 1). During the first eight weeks, between December 30 and February 21, samples were available from 12 cows, 6 in each group. There was a total of 22 quarters from which satisfactory samples were obtained from the cows receiving the timothy hay and grain ration and 23 among those in the other group. Beginning with the test of February 28, all 16 cows were included as the other 4 had freshened. This brought the total quarters up to 30 in the timothy hay group and 31 in the other group. The graph (fig. 1) shows the results obtained for the two 6-cow groups continuously throughout the experimental period and also for the groups after the 2 additional cows had been added to each group.

The average figures for the two groups were rather similar during the first eight weeks. On January 26 and February 2, unusually large quantities of sodium hydroxide were needed, which it was believed were due to variations made in conducting the test. Milk samples from the group receiving the better quality roughage (alfalfa hay and silage) consistently were less suitable on the average for acid production by *Str. agalactiae* than those from the group fed the poor quality hay beginning with the test conducted in the ninth week (February 28) and continuing for the remaining seven weeks of the experiment. For the eight tests made during this period, the average quantity of sodium hydroxide required to neutralize the acid in the samples from the 6-cow group fed timothy hay was 2.19 ml. and for the samples from the group receiving alfalfa

hay and silage 1.64 ml. The average quantities required for the groups with the 2 additional cows in each were 2.33 ml. in the case of those receiving the timothy hay and 1.69 ml. for the others. The average differences between the quantities of sodium hydroxide for the 6-cow groups was 0.55 ml. and with the extra cows 0.64 ml. which, respectively, represent 33.5 per cent and 37.8 per cent greater quantities required to neutralize the samples from the animals receiving the least desirable ration. Differences were observed in the milk of individuals within the groups that apparently were unrelated to the feed, and in the case of some animals variations existed between milk samples from different quarters.

The results were submitted to statistical analysis using Students t-test[5] by comparing the figures obtained on each test date. There were no significant differences between the figures obtained in the weekly tests for the 6-cow groups for the first 11 tests (December 30 to March 7). The differences were significant at the 1.0 per cent level on March 15, at the 5.0 per cent level on March 22, and at the 1.0 per cent level on March 29. They were insignificant on April 4, but were significant at the 5.0 per cent level the following week and again were insignificant on April 19. When all 8 cows were included in each group, the differences were insignificant for the first two tests (February 28 and March 7) and also on April 4. The differences were significant at the 1.0 per cent level on March 15, 22, 29, and April 12 and at the 5.0 per-cent level on April 19. The differences between the combined results for the last eight tests (February 28 to April 19), both in the case of the 6-cow groups and with 2 additional cows in each group, were significant at the 1.0 per cent level.

Incidence of Mastitis.—Two of the 8 cows in the group which received alfalfa hay and silage had slight manifestations of mastitis in one quarter each. One had a slightly swollen quarter (RR) which produced milk containing a little sediment in the foremilk on March 7 and 8. Later, on April 19, the pH of the milk sample from this quarter was observed to be abnormally high. A limited number of staphylococci were present in samples from this quarter. The other cow was shedding *Str. agalactiae* when placed on experiment. Increasingly stronger Hotis reactions in uninoculated tests were obtained until March 15, on which date the milk sample was excessively alkaline in reaction. This abnormality was detected in samples collected during the following three weeks but later they were normal in this respect. Two cows in this group had past histories of mastitis, but neither gave visible evidence of clinical mastitis during the experimental period. One had lost a quarter due to injury. The other had produced abnormal milk in each quarter at some time during previous lactations. She persistently shed *Str. agalactiae* from two quarters and staphylococci from one quarter.

Evidence of mastitis was manifested by seven quarters in 5 cows among the 8 in the group fed timothy hay for roughage.

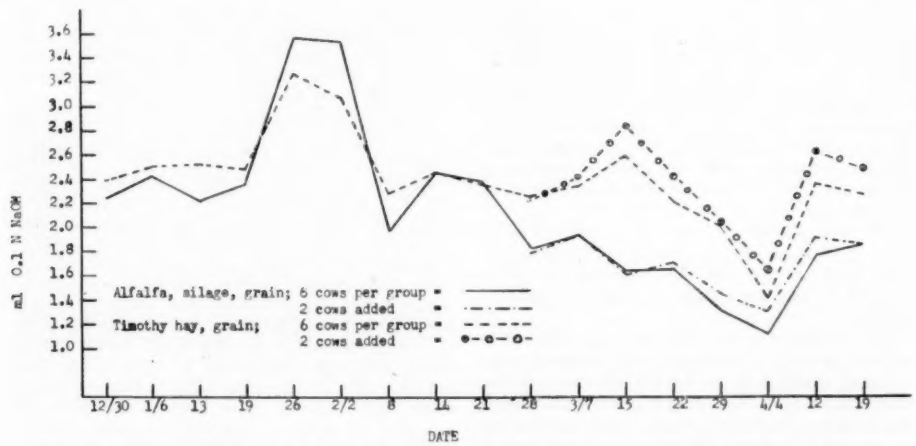

Fig. 1—Average quantities of sodium hydroxide required to neutralize the acid formed by Streptococcus agalactiae in milk samples from cows fed alfalfa, silage, and moderate quantities of grain, versus cows fed low quality timothy hay and heavy grain feeding.

The milk from one quarter on 1 of the cows, which freshened after the experiment was in progress, was noticed to be limited in quantity at freshening and samples were abnormally alkaline in reaction. This quarter produced only limited quantities of milk during the remainder of the experiment. Samples from one quarter of another cow were abnormally alkaline in reaction on March 22. Many samples, obtained from a third cow, were somewhat watery in appearance and of abnormally high pH, particularly during the later weeks of the experimental period. Both these latter cows had histories of mastitis associated with *Str. agalactiae* during previous lactations.

A fourth cow produced watery appearing milk containing numerous flakes or clots in one quarter for a week during the middle of April. During this period, a Streptococcus which did not produce acid reactions in Hotis tests was present in the samples from the affected quarter in considerable numbers. The fifth cow in the group to be affected had an acute attack in one quarter (LR) on March 5, at which time only serum and clots were obtained for two days. A Staphylococcus was present in the samples collected shortly after the attack. This cow had had an acute attack in one quarter (RR) associated with coliform organisms two years previously from which she made an uneventful recovery. *Streptococcus agalactiae* was detected for the first time in samples obtained from her on March 15, at which time they were present in milk from the right front quarter. This organism continued to be shed by this quarter throughout the remainder of the experiment.

Discussion

The observation that the milk from the animals which were consuming the better quality roughage and smaller quantities of grain apparently was more resistant, on the average, to the action of the streptococci is not of much significance unless a relationship exists with infection entry or clinical manifestations of mastitis. While extensive information could not be obtained in this limited study, it is of interest that the incidence of clinical cases was highest in the group receiving the poorer qualty roughage and larger quantities of grain, even though *Str. agalactiae* was not alone involved in these cases. Perhaps the beneficial influence on the health of calves of adequate proportions of good quality roughage in their diet[7] may be reflected in cows, such as increased resistance to mastitis through increased resistance of their mammary secretions to activity in them of some of the microörganisms which are pathogenic for the udder.

Summary

Milk samples collected at weekly intervals from two groups of 8 cows each were compared for suitability for acid production by *Streptococcus agalactiae*. One group received fairly good quality alfalfa hay, meadow crop silage, and moderate quantities of grain and the other group rather poor quality timothy hay and much heavier grain feeding. Brom cresol purple indicator was added to 10 ml. of quarter milk samples (Hotis test); these were inoculated with *Str. agalactiae* and following 24-hours incubation, 0.1 N sodium hydroxide solution was used to titrate the tests back to approximately their original color. The procedure was considered to offer a measure of the comparative resistance of the milk to activity of this mastitic infection and indirectly a possible measure of resistance of the animals to mastitis associated with this organism.

Beginning with the test conducted in the ninth week and continuing for the seven remaining weeks of the experiment, milk samples from the group receiving the alfalfa hay, silage, and moderate quantities of grain required on the average less sodium hydroxide to neutralize the acid formed than did the samples from the other group of cows. For the eight tests made during the last seven weeks, the average quantity of 0.1 N sodium hydroxide required for the samples from the group fed the timothy hay above that for the group receiving alfalfa hay and silage was 0.55 ml. in the case of 6 cows in each group which milked throughout the experiment and 0.64 ml. for the complete groups of 8 cows. These figures respectively represent 33.5 per cent and 37.8 per cent greater quantities. Differences were observed in the milk of individuals within the groups that apparently were unrelated to the feed.

Two cows in the group receiving alfalfa hay silage and grain had slight manifesta-

tions of mastitis in the quarter each during the experimental period. Evidence of mastitis was manifested by seven quarters on 5 cows among those receiving the timothy hay and grain.

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Variations in the Resistance of Milk to the Activity of Streptococcus Agalactiae

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IN A PREVIOUS STUDY, milk samples from a group of cows fed alfalfa hay, forage crop silage, and moderate quantities of grain were observed to be more resistant, on the average, to the production of acid in them by *Streptococcus agalactiae* than samples from a similar group of animals receiving poor quality timothy hay and heavier grain feeding.[7] The idea of exposing milk samples to the action of *Str. agalactiae* stemmed from efforts to find a method for measuring the relative resistance of animals to this organism without direct exposure of them. During the above study, it was evident that factors other than feed were responsible for some of the differences between samples from different animals, and between samples from the same individual at different times. By necessity, the investigation ended after less than 4 months duration.

The present report concerns observations made on milk samples from a number of animals in another herd over a period of a year. Emphasis was placed on the study of individual animals and several were followed through complete lactations.

EXPERIMENTAL

The same methods in general were followed as outlined previously.[7] However, only alfalfa and timothy hays and grain were used for feeding the cows. All animals had received timothy and clover hay prior to the start of the experiments.

An effort was made to pair off the majority of the cows into two groups, depending on their breed, age, and stage of lactation. The animals were of Holstein-Friesian and Guernsey-Jersey breeding. One group received no. 1 alfalfa hay, free choice, and a grain ration containing 16.0 per cent protein composed of corn, oats, bran, cottonseed meal, linseed and soybean oil meals, bone meal, and salt. The other group received timothy hay with some clover in it, at times, and a grain ration composed of similar feeds received by the first group but containing 18.0 per cent protein.

Animals fed timothy hay received approximately 1 lb. of grain for each 2.5 to 3 lb. of milk produced, whereas those on alfalfa hay were fed at the rate of approximately 1 lb. of grain for each 3 to 4 lb. of milk produced, but in no case was 14 lb. per day exceeded. The hay samples were collected weekly, grouped by months, and analyzed for protein and carotene content. The protein content of the alfalfa ranged between 10.4 and 19.99 per cent, with an average of 17.51 per cent. The protein in the timothy hay ranged between 5.52 and 8.64 per cent, with an average of 7.16 per cent. Carotene levels of the alfalfa varied from 9.5 to 30.4 μg. per gram and averaged 18.3 μg.; those of the timothy, between 2 and 5.5 μg. per gram with an average of 4.2 μg.

Quarter milk samples were collected aseptically approximately three hours after the morning milking once each week. Duplicate milk samples were prepared after the manner of the Hotis test.[1] One of each pair was inoculated with a 24-hour serum broth culture of *Str. agalactiae*. The culture was the same as the one used in the earlier study.[7] The stock culture was carried on blood agar. Preparation of the broth culture used for inoculating the milk samples was accomplished by transferring the culture to serum broth five days before it was to be used, repeating the transfer the next day and making the next and final transfer the day before use. Inoculation of the milk samples was carried out by use of a straight platinum wire. They were titrated back to approximate their original purple color after twenty-four hours of incubation at 37 C., using 0.1 NaOH sclution. The weekly milk samples were examined bacteriologically by plating directly onto blood agar.

RESULTS

Effect of Feed.—The weekly averages of the quantities of 0.1 N NaOH solution required to neutralize the acid formed in the milk samples from the animals fed the two types of hay were compared. Both types of hay were available from Nov. 15, 1950, to Sept. 10, 1951. During the first

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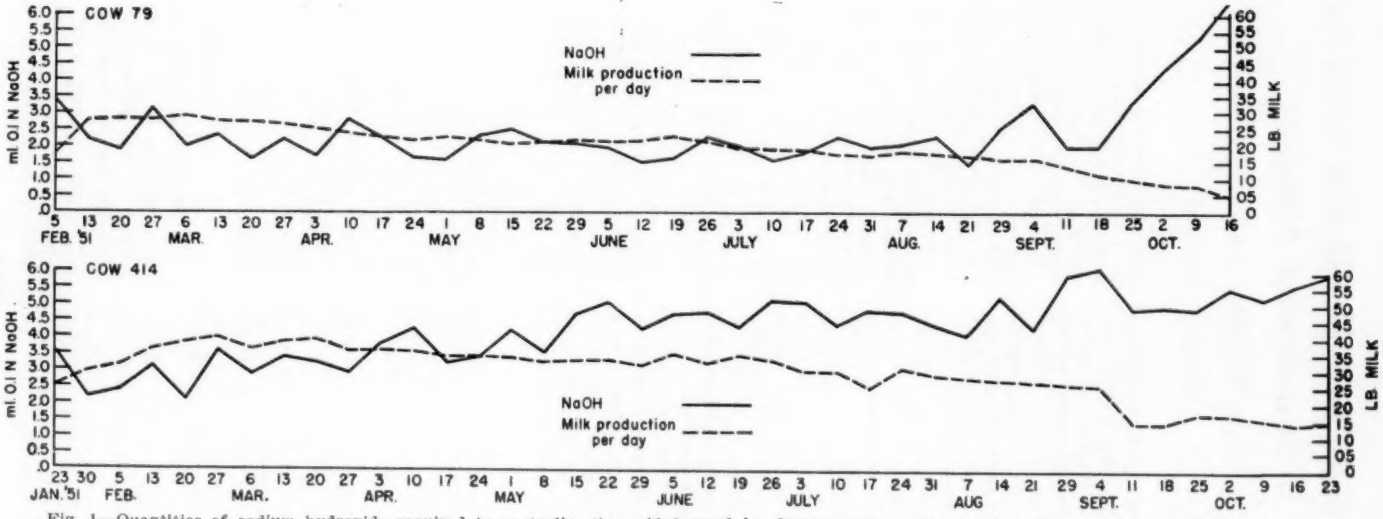

Fig. 1—Quantities of sodium hydroxide required to neutralize the acid formed by *Streptococcus agalactiae* in milk samples collected weekly during the lactations of 2 cows and their daily milk production.

ten weeks ending January 23, milk samples from the group receiving the alfalfa required a higher average quantity of NaOH to neutralize the acid resulting from activity of *Str. agalactiae* than those from the timothy group. The figures were 3.72 ml. for the former and 3.26 ml. for the latter. For the ten weekly tests beginning January 30, samples from the group fed alfalfa hay consistently required less NaOH solution to neutralize the acid than those from the others, the values being 3.03 ml. and 3.29 ml., respectively. There was little difference between the figures for the following five weekly tests. Then a repetition of the previous period occurred and for four tests, milk samples from the alfalfa group required less NaOH solution, the average amounts being 3.31 ml. and 3.61 ml. For the eight weekly tests conducted between June 12 and July 31, there was little difference between the averages, which were 3.75 ml. and 3.79 ml. Samples from the alfalfa group again required less NaOH at the time of the next three tests, the average values being 3.50 ml. and 3.81 ml. There were no differences between the groups for the following three weekly checks, the last of which was conducted on September 11 when alfalfa feeding was discontinued. During two of the first three weeks that both groups received timothy hay, near the end of the period of observation, the averages reversed. This was the first time that they had done so to any appreciable degree since the test of January 23 conducted more than seven months before. For the alfalfa and timothy groups the average figures for the whole period of observation were 3.55 ml. and 3.58 ml., respectively, and 3.38 ml. and 3.54 ml. for the period between January 23 and September 11.

The indication that the milk from the alfalfa hay group was a little more resistant to *Str. agalactiae* activity, once the animals had been fed this hay for several weeks, was not considered to be of much consequence as even the greatest weekly test difference during this period was less than 20 per cent. The results therefore did not reflect marked differences between the two rations in their ability to influence the resistance of the milk to the action of *Str. agalactiae*, such as was obtained when much greater extremes in rations were used.[7] Milk production of the animals receiving the alfalfa hay generally was higher than that of the animals getting timothy.

Effect of Age and Breed.—Comparison was made of the ability of various animals and groups to produce milk which was relatively resistant to the activity of *Str. agalactiae*. In making this comparison, note was taken of the first month after freshening in which the average quantity, for the month, of 0.1 N NaOH required to neu-

TABLE 1—Average Amount of 0.1 N NaOH Required to Neutralize the Acid Formed by Streptococcus Agalactiae in Milk Samples, Average Daily Milk Production, and Time That Visible Symptoms of Mastitis Were Present in Holstein-Friesian Heifers

Lactation-Month and Cow		1	2	3	4	5	6	7	8	9	10
400											
Fr. 2-18-51	NaOH (ml.)	3.18	3.35	3.20	4.05	4.18	3.99	5.13	4.68	4.80	(1 test)
Calf 1											
Timothy	Milk (lb.)	29.50	33.00	31.60	25.70	22.30	21.70	15.60	12.70	11.40	(1 week)
405											
Fr. 12-24-50	NaOH (ml.)	3.35	3.18	3.75	3.31	3.66	4.78	4.69	4.75	4.85	.....
Calf 1											
Timothy	Milk (lb.)	30.50	31.60	27.00	24.90	24.80	20.10	19.00	17.10	11.80	.....
406											
Fr. 3-24-51	NaOH (ml.)	2.86	2.07	3.57	3.56	3.47	4.29	4.05	.....	.....	.....
Calf 1											
Alfalfa	Milk (lb.)	36.10	37.00	34.30	31.40	30.10	25.10	21.50	.....	.....	.....
414											
Fr. 1-14-51	NaOH (ml.)	2.71	3.30	3.57	4.20	4.53	4.88	4.59	5.49	5.33	5.97 (1 test)
Calf 1											
Alfalfa	Milk (lb.)	32.20	38.70	35.70	33.80	33.80	29.70	28.60	20.20	15.90	13.0 (1 week)
	Mastitis (days)	0.0	0.0	1[a]	2[a]	1[a]	4[a]	11[a]	12[a]	4[ab]	0 (1 week)
188											
Fr. 5-2-51	NaOH (ml.)	3.77	4.42	3.65	4.76	4.90	4.50	.....	.....	.....	.....
Calf 2											
Alfalfa	Milk (lb.)	50.70	44.50	33.20	31.70	24.40	23.00	.....	.....	.....	.....
	Mastitis (days)	0.0	11[ac]	31[a]	12[a]	0.0	4[ab]	.....	.....	.....	.....

[a] = micrococci; [b] = *Str. agalactiae;* [c] = coliform.

tralize the acid formed in the samples was 4 ml. or greater, as 4 ml. seemed to be a suitable borderline. The data concerning one lactation each for 15 of the 22 animals are shown in tables 1, 2, and 3. Data for the remaining 7 animals, and parts of other lactations, are not presented in tabular form as they had but minor additional value because of the shortness of the period of observation or other limiting factors.

There were 9 first-calf Holstein-Friesian heifers and data for 4 are presented in table 1. Two of them (405 and 406) produced milk which on the basis of the 4-ml. level was relatively resistant to the production of acid until the sixth month of lactation, when their monthly averages first reached 4 ml. Two others (400 and 414) produced milk of similar resistance until the fourth month after freshening. Data began with the fourth month of lactation on another animal (188). From the available information, it appeared that her fifth month probably was the first in which an average of 4 ml. was reached. Two additional heifers also produced milk which was relatively resistant during a little more than their first two months after freshening, which was the extent of the period that they were observed. The remaining 2 heifers, which were also observed for only limited periods after freshening, were observed to produce milk which lacked resistance at first compared to the others, as in both instances monthly average figures in excess of 4 ml. were obtained.

Only 1 Holstein-Friesian (188) was observed during her second lactation. An average of more than 4 ml. NaOH was recorded for the second month after freshening (table 1).

As shown in table 2, among the 5 Guernsey-Jersey animals in their second and third lactations, only 1 (71) fairly early in the lactation period produced milk that supported activity of the streptococci to the extent that a monthly average of 4 ml. NaOH was required to neutralize the acid. This first occurred during the fifth month. Three others (47, 77, and 79) produced milk of similar characteristics but not until the end of their lactations nine months or more after freshening. The remaining 1 (87) never produced milk which averaged as much as 4 ml. NaOH per month during this lactation period.

There were 8 mature Holstein-Friesian cows and data on 5 are shown in table 3. All of them had histories of having had mastitis, caused by experimental inoculations, during previous lactations. Information was available on 2 for parts of two lactations each, making a total of ten lactations, or parts of lactations, to be included for this group. Average monthly quantities of NaOH of 4 ml. or more were recorded during the first month of their lactation in 2 animals (36 and 50), and in 1 (78) in the second month. Similar quantities were recorded in both lactations of 1 cow (6) during the third month, and in those of 3 cows (18, 78 and 805) during the fourth month after freshening. The remaining 2 (905 and 924) never produced milk which needed as much as an average per month of 4 ml. to neutralize the acid formed by the streptococci inoculated into it.

Thus the majority of the first-calf Holstein-Friesian heifers produced milk of relatively greater resistance during the earlier months of lactation than the 1 animal of similar breed in her second lactation and the older cows. The Guernsey-Jersey cows, during the earlier months of their lactations, produced milk which was even less suitable on the average for the activity of *Str. agalactiae* than that of the Holstein-Friesian heifers.

The trend of the NaOH figures was upwards, indicating lessening resistance of the milk as lactations progressed in the majority of the animals. This was true of all the Holstein-Friesian heifers that were followed during the major part of a lactation period with probably one exception (188) for which the data were incomplete. Three of the Guernsey-Jersey cows and 4 of the mature Holstein-Friesian cows followed the same pattern, even though some did so to a much less degree.

One Guernsey (79) and 2 mature Holstein-Friesians (18 and 50) had comparatively marked and sudden reduction in the resistance factors of their milk at the end of the lactation. Two others (47 and 77) in the Guernsey-Jersey group had a few unusually high readings for NaOH quantities during the last few weeks of the lactation. Production of milk per day was very low in both of the Holstein-Friesians, and in 1 of the Guernseys at this time. Two of these animals (50 and 79) were among those that maintained a steady, relatively

high resistance of their milk until the sudden loss occurred. Both animals also had been milked once per day during the last week of their lactations.

The two examples in figure 1 demonstrate some pertinent details such as weekly test variations, and differences between individuals in trends, as lactation progressed, of NaOH requirements for neutraliizing the acid produced in their milk samples and their milk production.

Influence of Milk Production.—The level of the resistance of milk samples to the activity of *Str. agalactiae* appeared to be related to some extent to the level of milk production of the animals (tables 1, 2, and 3). For one thing, the Guernsey-Jerseys were all comparatively poor producers and there was only 1 for which a figure of 4 ml. was obtained during the earlier months of her lactation. This one was the youngest of the 5, was one of the best and more persistent milkers, and the gradual loss of resistance in her milk was more pronounced than the others. For another, comparison of the heifer (188) which was observed during parts of her first two lactations showed that the lower resistance of the milk produced during the second lactation was accompanied by much greater production, at least during the earlier weeks and before she had mastitis. Then again, the heaviest producer among the first-calf heifers (414) had the earliest and largest gradual loss in the resistance of her milk as her lactation progressed. She had been further handicapped because her calf had to be removed by cesarean section. Comparison of the effects of milk production on the resistance of the milk of the older cows was of limited value because of their previous exposure to mastitis. This undoubtedly hampered the production of some of them. The 1 animal (50) of this group which did not produce visibly abnormal milk maintained a rather even and moderate level of resistance of her milk and was also a rather poor milk producer.

TABLE 2—Average Amount of 0.1 N NaOH Required to Neutralize the Acid Formed by Streptococcus Agalactiae in Milk Samples, Average Daily Milk Production, and Time That Visible Symptoms of Mastitis Were Present in Guernsey-Jersey Cows

Lactation, Month and Cow		1	2	3	4	5	6	7	8	9	10	
47	NaOH (ml.)	2.38	3.41	2.70	3.26	3.36	3.31	2.48	3.41	3.95	4.35	(2 tests)
Fr. 1-10-51	Milk (lb.)	35.10	32.70	28.30	24.20	19.80	19.90	20.90	16.80	16.00	15.00	(2 weeks)
Calf 2 Timothy	Mastitis (days)	0.0	3[a]	0.0	1.00	0.0	0.0	0.0	0.0	1.00	1.00	(2 weeks)
71	NaOH (ml.)	1.75	2.68	2.74	3.02	4.05	4.11	4.20	4.19	4.87	.....	
Fr. 1-29-51	Milk (lb.)	29.90	27.30	26.70	25.00	24.60	22.20	21.50	16.50	13.80	.....	
Calf 2 Alfalfa	Mastitis (days)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4[a]	.....	
77	NaOH (ml.)	2.68	3.12	2.53	3.75	3.53	3.42	3.71	3.46	4.87	.....	
Fr. 1-26-51	Milk (lb.)	22.90	17.80	15.00	15.60	13.40	11.60	11.00	6.70	5.40	.....	
Calf 3 Timothy	Mastitis (days)	1.00	27[b]	1[b]	4[b]	4[b]	1[b]	0.0	0.0	0.0	.....	
79	NaOH (ml.)	2.69	2.01	2.12	2.30	1.93	1.95	2.36	2.92	5.86	.....	
Fr. 2-2-51	Milk (lb.)	20.40	27.00	22.60	21.30	21.20	18.20	17.40	10.70	6.5	.....	
Calf 3 Alfalfa	Mastitis (days)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4[c]	.....	
87	NaOH (ml.)	2.27	2.22	2.50	2.48	2.33	2.64	2.73	3.45	3.64	.....	
Fr. 1-14-51	Milk (lb.)	29.60	24.20	17.50	17.40	13.80	10.50	9.50	7.30	6.10	.....	
Calf 3 Timothy												

[a] = micrococci; [b] = hemolytic Staphylococcus; [c] = atypical Streptococcus.

CLINICAL AND ASSOCIATED BACTERIOLOGICAL OBSERVATIONS

Holstein-Friesian Heifers (First and Second Calves).—Clinical cases developed in 3 of these animals. One of them was under observation for only the first three months of her lactation and during this period her milk lacked resistance to acid production by *Str. agalactiae.* For six days immediately after freshening her udder was comparatively firm and flakes were present in the foremilk. Bacteriological examinations showed the presence of micrococci in the samples from the affected quarter.

In the second heifer (414), all four quarters were involved. She produced foremilk containing some flakes and sediment on 23 occasions between the third and eighth months after freshening (table 1). In the latter month, she also suffered a moderately acute attack in the left front (LF) quarter with swelling and milk changes that persisted for a week. She had a second, less severe attack during the following month in the right front (RF) quarter. Micrococci and diptheroids were the only bacteria isolated from her samples until the tenth month and these were persistently present before abnormal secretions were first detected. Early in the tenth month, *Str. agalactiae* was recovered from the right rear (RR) quarter. The resistance of her milk to *Str. agalactiae* activity gradually decreased during the lactation (fig. 1). From the fourth month on, there were several instances when the weekly NaOH figures reached 5.0 ml. A couple of days before the attack near the end of her eighth month of lactation, a reading of 6.15 ml. was obtained. The week previous it was 5.90 ml. During the period when the second acute attack occurred, the readings again were between 5.0 and 6.0 ml.

The heifer (188) which was observed during parts of her first two lactations had a total of eight days during the last five months of her first lactation when a few flakes were present in foremilk samples. During this period, the resistance factors for activity of *Str. agalactiae* in her milk had gradually improved and it was not possible to note a relationship between the test figures and the times when the flakes were present. The only organisms isolated from her milk samples were micrococci and diptheroids. Clinical symptoms were absent during the first eight weeks after she freshened the second time, but she then suffered a severe sudden attack in the RF quarter. A few colonies of coliform organisms were the only bacteria obtained from

TABLE 3—Average Amount of 0.1 N NaOH Required to Neutralize the Acid Formed by Streptococcus Agalactiae in Milk Samples, Average Daily Milk Production, and Time That Visible Symptoms of Mastitis Were Present in Mature Holstein-Friesian Cows

Lactation-Month and Cow		1	2	3	4	5	6	7	8	9	10
6	NaOH (ml.)	NR	2.36	4.23	3.33	2.89	3.86	2.91	3.15	3.64	4.44 (2 tests)
Fr. 8-27-50	Milk lb.)	NR	42.70	30.80	27.10	23.00	19.60	17.80	14.70	8.80	6.60 (2 weeks)
Calf 4 Alfalfa	Mastitis (days)	NR	NR	31[ab]	29[ab]	31[ab]	31[ab]	28[a]	26[a]	15[a]	7 (2 weeks)
18	NaOH (ml.)	2.76	3.42	3.46	4.53	4.60	4.40	5.10	4.98	5.64 (2 tests)	
Fr. 2-10-51	Milk (lb.)	21.10	22.10	21.30	21.10	17.00	14.00	11.40	10.00	7.00 (2 weeks)	
Calf 4 Timothy	Mastitis (days)	28[ab]	12[ab]	1[ab]	0	0	30[ab]	21[ab]	5	13 (2 weeks)	
36	NaOH (ml.)	4.15	3.81	4.05	3.31	3.33	4.03	4.89	4.36 (1 test)		
Fr. 3-17-51	Milk (lb.)	56.00	61.40	50.20	38.80	31.00	26.60	20.90	20.00 (1 week)		
Calf 5 Alfalfa	Mastitis (days)	31[ac]	20[ac]	31[ac]	31[a]	30[a]	17[a]	16[a]	7 (1 week)		
50	NaOH (ml.)	4.11	3.14	3.09	3.95	3.35	3.28	3.74	3.63	5.78 (2 tests)	
Fr. 11-27-50	Milk (lb.)	39.00	40.00	31.70	25.50	20.50	17.40	13.50	8.00	2.00 (2 weeks)	
Calf 3 Timothy											
805	NaOH (ml.)	3.13	3.00	3.85	4.15	4.03	3.90	4.41	4.28	4.39	5.36 (1 test)
Fr. 11-30-50	Milk (lb.)	34.00	41.70	39.00	36.80	34.20	32.00	28.90	22.40	19.00	17.50 (1 week)
Calf 6 Alfalfa	Mastitis (days)	30[a]	31[ac]	30[a]	29[ac]	27[a]	30[a]	31[c]	30c	31[c]	7 (1 week)

NR = no record; [a] = *Str. agalactiae;* [b] = *Str. dysgalactiae;* [c] = *Str. uberis.*

samples from the affected quarter when inoculated directly onto blood agar. Prior to the attack, micrococci frequently were present in samples from this quarter. Milk production which had been in excess of 50 lb. per day at the time of the attack dropped to half this quantity but slowly increased to 35 lb. The resistance factors for activity of *Str. agalactiae* in her milk had gradually decreased so, that by the end of the seventh week after freshening, the average quantity of NaOH solution required to neutralize the acid formed in the quarter samples was 5.15 ml. The figures obtained during the previous weeks were 4.35, 3.45, 3.40, 3.30, 4.30, 3.85, and 4.75 ml. The attack gradually subsided but mild clinical symptoms such as flakes in the milk were present for the next six weeks. The quantities of NaOH required for neutralizing the acid formed in the tests tended to be lower during the next several weeks after the attack and the sharp drop in milk production, but they began to increase later. For four days during the sixth month flakes again were present in foremilk samples. During this period, *Str. agalactiae* was recovered from samples from the previously affected quarter (RF) and from another left rear (LR).

Guernsey-Jerseys (Second and Third Calves).—One animal (87) had no manifestations of mastitis. Another (47) produced milk which contained a few flakes on six occasions, two of these being at the end of her lactation when two unusually high NaOH readings for her, namely, 5.0 and 5.7 ml., were obtained. A Micrococcus was present in the abnormal samples and in the samples prior to this. A third (79) produced milk having a few flakes during the last week of her lactation when she was being milked once per day and at the time when the resistance factors in her milk had just taken a sharp drop (fig. 1). An atypical Streptococcus was present in the samples from the affected quarter. A fourth animal (77) had some flakes in foremilk samples on a total of thirty-eight days, most of them during the second month of the lactation. She persistently shed from the affected quarters hemolytic staphylococci that were coagulase-positive. A relationship was not noticeable between the resistance factors of her milk for *Str. agalactiae* activity and the occurrence of the abnormal milk. The fifth cow (71) had a mild attack in the RF quarter which persisted for four days during her ninth month of lactation. This occurred during the week following the first time that the quantity of NaOH required to neutralize the acid formed by *Str. agalactiae* in her milk had reached 5.0 ml. Micrococci were present in samples from this quarter prior to the attack and also in the abnormal milk samples.

Mature Holstein-Friesians.—All 8 of the mature animals had been experimentally exposed to *Str. agalactiae* or other mastitis infections during previous lactations with the result that all had diseased conditions of their udders of varying severity. One of them (50) gave no visible manifestations of mastitis throughout the lactation, although there were some small but definite areas of fibrosis in her udder and she shed *Str. agalactiae* in moderate numbers at all observations. Quantities of NaOH used in the weekly resistance observation of the milk seldom were as high as 4 ml., and never were more than slightly above this figure except during the last two weeks when production was very low and an extreme rise occurred.

The average NaOH figures for two (924 and 905) were relatively moderate but on a number of occasions during the later months of their lactations, more than 4 ml. was obtained, sometimes as much as 4.7 ml. for weekly tests. During the later months, visibly abnormal foremilk samples were obtained from both animals on numerous days. *Streptococcus agalactiae* was shed by both and also *Streptococcus dysgalactiae* by cow 905.

Four cows (6, 36, 78, and 805) which had freshened four to six times each suffered from mastitis of varying degrees most of the time during the period of observation. All received treatments with antibiotics at various times. Readings above 4 ml. were frequent and many were up to 5 ml. All shed *Str. agalactiae,* 3 shed *Streptococcus uberis;* and 1, *Str. dysgalactiae.* As lactation progressed, the upward trend in quantities of NaOH required to neutralize the acid formed in milk samples by *Str. agalactiae,* which was frequently observed in the younger animals, was not apparent or only in slight degree in these 4 cows. In another cow (18), however, this trend was observed in samples obtained during her third and fourth lactations. A sharp rise in NaOH occurred as she went dry at the end of her third lactation, at which time readings in excess

of 5 ml. were obtained. A small quantity of flakes was present in foremilk samples on one occasion during the last month and *Str. agalactiae* was recovered a number of times. When she freshened for the fourth time, abnormal milk which was associated with *Str. agalactiae* and *Str. dysgalactiae* was produced from the beginning for over a month. Only moderately low readings for NaOH were obtained at this time and it was not until the fourth month that more than 4 ml. was recorded. She recovered from the clinical symptoms without treatment prior to this but three months later had recurrences. There was a marked trend upwards in NaOH throughout this lactation until as much as 6 ml. was recorded. This occurred even though milk production was low. This was similar to the trends in quantities of NaOH as seen in the younger animals but in them it appeared that marked upward trends accompanied heavier and persistent production.

DISCUSSION

The influence of an inheritance factor on the resistance of milk to *Str. agalactiae* which cows could maintain might be expected. Possibly the present data lends some support for the idea because of the difference between individuals, particularly during the few weeks after their first freshening, and the breed difference encountered in animals under the same general environment.

The level of milk production seemed to offer a reasonable explanation for some of the variations observed in resistance of the milk even though there were a few exceptions such as was provided by the record of cow 18. Her milk production dropped off rather markedly as did the level of resistance. There were no instances of the opposite trend, that is, of excellent production with greatly retained resistance so perhaps her record was influenced by other factors.

The data obtained in the previous experiment was the strongest indication that feed variations can play a part in resistance. Should the quality and quantity of roughage ingested in proportion to the intake of grain be a factor, then the failure to obtain a sizeable difference between the groups of animals on the two types of roughage in the present report needs an explanation. Perhaps this may be provided by the fact that the milk production was stimulated to greater levels by the better quality roughage and thus greater demands followed for resistance factors. Although the alfalfa and grain diet was able to support levels of resistance factors equivalent to those in the milk of the animals on the other diet composed of timothy hay and grain, it was unable to exceed them such as apparently occurred in the previous experiments in which meadow crop silage was included in the ration with the alfalfa. The causes of the differences obtained between the average NaOH figures for the animals making up the present herd and the previous one[7] have many possible origins, but data to explain them adequately are not available. The herds had received different treatment prior to the experimental periods and they were fed and managed differently. Although the same culture of *Str. agalactiae* was used in both studies, equipment and personnel differed somewhat and some variations were incorporated in the laboratory procedures.

Some indication was obtained that age was involved as the heifers, for the most part, had better resistance in their milk during the earlier half of their lactations than the older cows of the same breed. Other workers have observed a comparatively high resistance of heifers to *Str. agalactiae* infections.[2, 4, 6] For several of the heifers a period elapsed between freshening and the first months when less satisfactory resistance of the milk was obtained, that was rather similar in length to the period after freshening before the first infections occurred in the experiments of Lancaster and Stuart.[4]

The sharp drop in resistance of the milk of some animals at the end of their lactations appears comparable to the observation of McEwen and White.[5] They observed a marked loss of the bacteriostatic property of the milk of a cow going dry when using *Str. pyogenes* as the test organism.

Care was taken to prevent the spread of *Str. agalactiae* from the infected animals to the heifers and Guernsey-Jersey cows by use of the usual procedures of sanitation and milking order. This could account, at least in part, for the failure of this organism to gain entrance to any quarters of the animals which had been experimentally exposed in previous lactations until late in the experimental period and then only in

three quarters of 2 animals and at times when the resistance of their milk was low.

The clinical cases, that occurred during times when resistance of the milk was low in the heifers and cows that had not previously been exposed experimentally to *Str. agalactiae,* seemed to involve micrococci, an atypical Streptococcus, and possibly a coliform. Even though the measure of the resistance is based on that obtained with *Str. agalactiae,* it is possible that the same or closely related factors restrain these other organisms because bacteriostatic properties of milk appear to be nonspecific to some extent.[3] The hemolytic Staphylococcus, present in a quarter of 1 of the Guernsey-Jerseys, was associated with mild clinical symptoms, yet the milk of this animal did not have low level of resistance to *Str. agalactiae.* This possibly indicates that a higher level of resistance factors in the milk was needed to inhibit this Staphylococcus adequately, or that specific inhibitors for this Staphylococcus were present in inadequate quantities and that they were not measured by use of *Str. agalactiae.*

SUMMARY

Quarter milk samples were collected aseptically at weekly intervals during a year from a herd comprised of 17 Holstein-Friesian and 5 Guernsey-Jersey cows. Brom cresol purple was added to 10-ml. quantities (Hotis test) and they were inoculated with *Streptococcus agalactiae.* Following twenty-four-hours-incubation, the quantity of 0.1 N NaOH was determined that would neutralize the acid formed and return the samples to approximately their original color.

During much of the period, half the herd received alfalfa hay and grain and the other half was fed timothy hay and grain. There was no appreciable difference between these two groups in the average resistance of the milk samples to the action of *Str. agalactiae.*

The majority of the 9 first-calf Holstein-Friesian heifers produced milk of relatively greater resistance to the activity of *Str. agalactiae* during the earlier months of lactation than the 1 animal of similar breed in her second lactation and the majority of the 8 older cows of the same breed. The 5 Guernsey-Jersey cows, which were all in their second or third lactations, produced milk which on the average was even more resistant than that of the others during this same stage of lactation.

In the majority of the animals, the trend as lactation progressed was towards a lessened resistance of the milk. In 3 animals, comparatively marked and sudden reduction of the resistance factors in the milk occurred as they reached the end of their lactations.

There was some tendency for heavier and more persistent milk production on the same ration to be accompanied by comparatively lower levels of milk resistance.

Three of the heifers developed clinical symptoms. These symptoms were associated with micrococci and possibly coliform organisms in one animal. At the time of the symptoms the resistance factors for *Str. agalactiae* in their milk were relatively low.

Four of the Guernsey-Jerseys manifested symptoms of mastitis during the period of observation. One was infected with hemolytic staphylococci, and the mild clinical symptoms occurred during a period when the resistance of her milk to *Str. agalactiae* was relatively high. Three had symptoms associated with micrococci or atypical streptococci towards the end of their lactations when resistance factors in their milk for *Str. agalactiae* were relatively low.

Only 1 of the mature Holstein-Friesians, all of which had been exposed experimentally during previous lactations to *Str. agalactiae,* did not have clinical symptoms of mastitis. She maintained a relatively even and moderate level of resistance in her milk but persistently shed *Str. agalactiae.*

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Reservoirs of Infection of Micrococcus Pyogenes in Bovine Mastitis

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THE SEARCH FOR adequate control measures for staphylococcal mastitis depends on basic information concerning its modes of transmission and its pathogenesis. This work was designed to establish the incidence and location of the organism on the skin and in the environment of the cow, and to compare staphylococci found in the environment and in aseptically collected milk samples from the same cows.

REVIEW OF THE LITERATURE

Micrococci have been recognized for a long time as common inhabitants of the interior of the mammary gland. Jones[1] in 1918 reported that the principal sources of micrococci in mastitis were apparently normal cows which had udder infections and that the organisms could be carried readily on the hands from cow to cow. Evans[2] found staphylococci in milk which resembled culturally those from skin and other sources. Staphylococci have been found in dermal abscesses of mammary skin[1, 3] and in other pyogenic lesions elsewhere in cattle. Miller and Heisman[4] suggested that staphylococcic infection can occur by way of the teat canal and that cows may get infected from men milking them. Schalm and Ormsbee[5] emphasize the milking act as an important factor in the spread of staphylococcic infection. They found that the incidence of infection increased with age, and that complete segregation reduced the spread of infection.

Slanetz *et al.*[6] made a study of cultures of staphylococci from human infection and from milk of cows affected with mastitis and found the organisms to be similar. They reported also that the coagulase test and hemolysis were useful criteria for virulence of staphylococci for the bovine mammary gland. Many different species of micrococci are found in aseptically collected milk and criteria for differentiation of pathogenic and nonpathogenic strains have been studied by other workers. Plastridge and co-worker[7] reported that the germentation of mannitol, hemolysis of ox blood, and the formation of coagulase were *in vitro* properties correlated to ability to cause mastitis than were other biochemical properties. Recently Barber and Kuper[8] found that phosphatase production closely paralleled coagulase production in numerous strains of staphylococci.

MATERIALS AND METHODS

The cows examined in this study were in a dairy herd of purebred animals of different breeds. The cows were held in one barn and milked in succession by two attendants using three machines. Before milking, the udders and teats were wiped off with a separate towel moistened in a sodium hypochlorite solution (about 300 parts chlorine per million, p.p.m.) and one or two streams of milk were removed into a strip cup. After milking each cow, the teat cups of the machines were dipped successively into two pails of chlorine solution before being placed on the next cow. The solutions were prepared to contain about 300 p.p.m. of chlorine and were changed once near the midpoint during the course of milking 62 cows. At the end of the milking period, the cups were rinsed with water and soaked in 0.5 per cent solution of sodium hydroxide until the next milking.

No precautions were taken to milk the cows in order of age or freedom from infection. Staphylococcic mastitis had been a continuous problem for many years in this herd. No infections with *Streptococcus agalactiae* were discovered during this work.

The presence of micrococci in aseptically collected milk samples was established on end milk collected shortly after the completion of milking. The udders were wiped off with a paper towel moistened in chlorine solution, and the ends of the teats were disinfected with a pleget of cotton moistened in 70 per cent ethyl alcohol. About 15 ml. were milked into a sterile container and taken to the laboratory. Samples were streaked onto phenol red-mannitol-salt agar plates.[9] After twenty-four and forty-eight hours' incubation at 37 C., the plates were examined and colonies picked for further identification. Blood-agar plates, various carbohydrates, and the coagulase test were utilized for further study of the strains isolated.

Samples for detection of micrococci in the environment were taken with sterile cotton swabs moistened in nutrient broth containing 4 per cent NaCl. The swabs were rubbed several times across the surface to be examined and then were returned to the broth. The seven sites sampled for each of 62 cows in the herd

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were the skin on the side of each of the four teats, the hair on the anterior surface of the leg, the vulva, and the floor of the stall beneath the cow's udder. Repeated swab samples were also taken in the teat cups of the milking machine just before milking, after milking, and after dipping the teat cups. A few samples for culture of the disinfectant solution used for towels and for dipping teat cups were taken. When samples were obtained in the presence of the chlorine disinfectant, the swabs were returned to 10 ml. of sterile skimmed milk or 10 ml. of 2 per cent sodium thiosulphate to inactivate the chlorine present. Both of these methods made possible the recovery of staphylococci from recently dipped teat cups, but the milk appeared to be superior so it was used after the preliminary trials.

After collection of the samples, the swabs were allowed to stand in the nutrient salt broth or milk for one to three hours. The swabs were then rubbed over the surface of a phenol red-mannitol-salt agar plate. The broth or milk remaining was incubated along with the agar plate for twenty-four hours at 37 C. A loopful of the broth or milk was then streaked over the surface of another salt-agar plate. At twenty-four and forty-eight hours, the plates from both the original and enriched culture were examined for the presence of mannitol fermenting staphylococci. Representative colonies were streaked onto the surface of Difco blood-agar medium containing 8 per cent bovine blood to determine the degree and type of hemolysis. Hemolytic cultures were examined for coagulase production with rabbit plasma by the tube method.

Nutrient agar containing 0.02 per cent sodium phenolphthalein phosphate was employed to demonstrate phosphatase activity. For primary isolations, this medium was made more selective by the addition of 7½ per cent sodium chloride. After incubation for twenty-four hours, cultures on ordinary phenolphthalein disphosphate agar or after forty-eight hours of incubation on the same agar containing sodium chloride, the covers of the Petri dishes were removed and the agar cultures were inverted over a 28 per cent solution of ammonium hydroxide. Phosphatase-producing colonies became pink and could be picked for subsequent biochemical tests and microscopic examination. Eight cows were reëxamined by cultures of milk, and swab cultures of skin, hair, vulva, and floor, using the sodium phenolphthalein phosphate salt agar for primary isolation.

A comparison of the resistance of cultures of micrococci to a sodium hypochlorite disinfectant was made. In these trials, sterile cotton swabs were dipped into a broth culture of a strain, immersed for ten seconds in the hypochlorite disinfectant and then placed in sterile skimmed milk for subsequent plate counts to determine the number of organisms surviving.

The persistence of a coagulase positive, hemolytic culture of micrococci on dry wheat straw was tested. The organism, which had been isolated recently from a case of mastitis, was grown twenty-four hours in skimmed milk; and 0.2 ml. of the culture containing about 14 million organisms was inoculated into each of numerous test tubes containing approximately 0.5 Gm. of straw. Part of the straw samples had been sterilized previously by autoclaving. Tubes containing the straw and milk culture were stoppered with an ordinary sterile cotton plug. Numerous tubes of sterilized and nonsterilized straw containing the culture were placed at each of the following temperatures: —20 C., 7 C., 21 C., and 37 C. At three, five and seven days, and weekly thereafter for forty-nine days, the number of surviving micrococci were determined by surface-plating dilutions on blood agar and phenol red-mannitol-salt agar.

RESULTS

Table 1 presents the results of cultures made on the cows and on their immediate environment. Many micrococci which would grow in salt agar and ferment mannitol were found on the skin of the teats, but the majority were nonhemolytic and only 12 were coagulase positive. The teat cups prior to disinfection were usually contaminated with mannitol-fermenting micrococci, but less than half of the cultures were hemo-

TABLE 1—Incidence of Micrococci on, and in, the Environment of Dairy Cows in an Infected Herd of 62 Cows

Location	No. examined	Number of samples with micrococci positive		
		Mannitol fermentation	Hemolysis	Coagulase
Skin of teats	248	194	77	12
Hair of flank	62	62	12	0
Vulva	62	12	4	0
Floor of barn beneath a cow	62	47	4	0
Teat cups before milking period	12	0	0	0
Teat cups before disinfection	124	102	47	15
Teat cups after disinfection	124	57	43	26
Hands before milking period	2	2	1	0
Hands during milking period	4	4	0	0
Disinfectant for teat cups	36	10	6	2
Aseptically collected milk samples	248	52	59	19

lytic and about 12 per cent were coagulase positive. After being dipped in a disinfectant, fewer cups were contaminated with mannitol-fermenting micrococci; but of those organisms recovered, 75 per cent were hemolytic and 46 per cent were positive for coagulase. In two instances, coagulase positive organisms were actually recovered from the disinfectant which had been used for dipping 17 and 32 sets of teat cups. In these results, the principal extramammary reservoir for hemolytic and coagulase positive micrococci appeared to be the skin of the teats and the teat cups of the milking machine. Further cultures using other methods for isolation resulted in similar findings.

TABLE 2—A Comparison of the Presence of Hemolytic and Coagulase-Positive Micrococci on the Skin of the Teats of Quarters with and Without Hemolytic Micrococci in the Milk

Milk cultures disclosed micrococci		Cultures of skin teat	
Hemolysis	Coagulase	Hemolysis	Positive coagulase
—189**		—132	
		+ 57	5
	—40	—36	1
+ 59*	+ 19	+ 23	5

*+ hemolytic on ox blood agar or positive on production of coagulase and phosphatase.
**— nonhemolytic on ox blood agar or no coagulase produced.

Table 2 compares the incidence of hemolytic and coagulase positive micrococci on the skin of the teats of quarters, with and without a hemolytic micrococcic infection. Cows often had hemolytic micrococci on the skin of the teats when none were found in the milk.

The results of a comparison of phosphatase with hemolysin and coagulase production by 186 cultures of micrococci isolated from milk or the environment of dairy cattle are presented in table 3. These cultures were selected originally for hemolysis (68) or phosphatase production (118), so that all were positive for one or the other of those characteristics. All 94 coagulase-producing cultures and 96 of 111 hemolytic cultures formed phosphatase, but many other micrococci also did so. The phosphatase-detecting medium was improved by the addition of 7½ per cent sodium chloride. Even so, it was apparently inferior to blood agar or phenol red-mannitol-salt agar for the isolation of micrococci from contaminated materials.

TABLE 3—Hemolysis, Coagulase and Phosphatase Activity of 186 Cultures of Micrococci

Hemolysis	Production of coagulase	Production of phosphatase
+ 111*	+ 92	+ 92
	—19	+ 4
—75**	+ 2	+ 2
	—73	+ 73

*+ hemolytic on ox blood agar or positive on production of coagulase and phosphatase.
**— nonhemolytic on ox blood agar or no coagulase produced.

Preliminary results indicated that coagulase-positive cultures were usually more resistant to hypochlorite disinfectant than were coagulase-negative cultures of micrococci isolated from aseptically collected milk samples. These results are being investigated further.

Coagulase-positive micrococci died rapidly at 37 C. in untreated straw, so that only 0.2 per cent were found alive after three days. Thereafter at intervals up to forty-nine days, the organisms were recovered in numbers varying from 0.1 up to 13 per cent. In sterile straw held at 21 C. or 37 C., no organisms could be recovered at three days or thereafter. In untreated straw at 21 C., organisms were recovered for the forty-nine-day period in numbers varying from 12 to 200 per cent of the number added. Death rates of organisms in straw held at 7 C. and —20 C. were very slow, and more than 10 per cent of them were still alive after forty-nine days. These results indicated that many coagulase-positive micrococci persisted in untreated straw at ordinary temperatures for forty-nine days. Death rates were more rapid at higher temperatures and in autoclaved straw.

CONCLUSIONS

Under the conditions of the observations, the principal extramammary reservoirs for coagulase-producing hemolytic micrococci discovered were the skin of the teats and the teat cups of the milking machine. Disinfection of teat cups by dipping in two successive pails of sodium hypochlorite solution containing chlorine was shown to be ineffective in many cases for complete elimination of contamination. Hemolytic and

coagulase-positive micrococci appeared to resist disinfection with hypochlorite better than other micrococci. Many of the organisms remained alive on dry straw for forty-nine days.

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Observations on the Clinical Features and Pathology of Hemolytic Icterus of Newborn Foals

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THE CONCEPT that a form of postpartum hemolytic anemia and icterus of newborn Equidae was due to an isoimmunization of pregnancy appears to have been developed and investigated independently and concurrently for mule foals in France and for horse foals in the United States and England.

From information available to the writer, the first reported demonstration that anemia and icterus of newborn mules was due to an isoimmunization against fetal red blood cells was that of Caroli and Bessis[1] in 1947. A report by Bruner, Hull, Edwards, and Doll[2] in 1948 called attention to an isoimmunization during pregnancy as the cause of hemolytic anemia and icterus of 6 newborn foals during the 1947 foaling season. Also in 1948, Coombs *et al.*[3] and Parry *et al.*[4] studied cases of icterus of foals in England and demonstrated that an isoimmunization of the mare was responsible for appearance of anemia and jaundice in the newborn foal.

Later reports[5-8] described the postnatal pathogenesis of hemolytic icterus, serological findings, and methods employed in prevention and treatment. The maternal antibody was obtained only through the colostrum after birth of the foal and severity of the disease was related to the antibody titer of the dam's serum and colostrum. Procedures employed in laboratory diagnosis were described by Bruner.[9]

CLINICAL OBSERVATIONS

Except for cases complicated by prenatal streptococcic infection, delayed parturition or dystocia, the foals appeared healthy at birth. They got up soon after birth, nursed strongly, and were active. The early symptoms of hemolytic icterus appeared to have escaped observation in several instances. In cases originating from known naturally immunized mares carrying incompatible foals which were permitted to nurse the mare, and in first cases for which a diagnosis was established during the first twenty-four hours, icterus was very slight or absent. From twelve to twenty-four hours after birth, some foals showed weakness and dullness and spent much time lying down. The conjunctiva, sclera, and mucous membranes were very pale. They nursed infrequently and then only for short intervals. At rest, the heart rate and the frequency and volume of respirations were increased. Exercise induced a rapid acceleration of the heart and respiratory rates, resulting in pulse frequencies of 150 to 180 per minute and respiratory cycles of 50 to 70 per minute. Citrated blood samples showed a marked reduction of erythrocyte content and the red blood cells usually were agglutinated in their own plasma. Erythrocyte counts on these foals ranged from 2,200,000 to 3,600,000 per cubic millimeter. In these cases, icterus was present on the second day and it became more intense on the third day and later. Also, the general symptoms often became more pronounced. Hemoglobinuria was seldom observed during the first twenty-four hours after birth but was present in a few foals on the second and third days.

Less severe cases were observed during the first twenty-four hours. These foals showed no definite symptoms of illness and the anemia was discovered by the farm veterinarian upon routine examination of the foals; also foals of known naturally immunized mares developed clinical anemia within twelve to sixteen hours without showing symptoms of illness. These foals had red blood cell counts ranging from 4,500,000 to 6,000,000/cmm. Some foals that were mildly affected on the first day showed icterus, severe anemia, and aggravated general symptoms on the second day or later. In these, the red blood cell count was lower, having fallen to 3,000,000/cmm. or less. It appeared that it was this type of foal that was observed by the stable attendant to be acutely affected for the first time on the second to fourth day after birth.

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In some foals, anemia and icterus apparently developed gradually over a period of several days without any outward symptoms of illness. In these, diagnosis was established by serological methods after the farm veterinarian had found slight or moderate icterus and anemia of the conjunctiva and sclera upon routine inspection of the foal. Red blood cell counts of these foals were between 6,000,000 and 7,000,000/cmm. Except for tiring rapidly during vigorous exercise, these foals have not shown symptoms of illness and they have not required treatment. This mild form of hemolytic icterus was diagnosed in several foals 4 to 9 days old.

Usually, the appetite was not affected. Foals maintaining red blood cell counts of 4,000,000/cmm. and higher usually nursed the mare at frequent intervals for short periods. Foals with red cell counts below 3,000,000/cmm. nursed infrequently. Following an exsanguination transfusion, nearly all foals would go to the mare and nurse strongly.

In the initial studies on this disease, it was difficult to account for the apparent rapidly fatal illness in some foals 4 to 6 days old. Stable attendants reported that no previous illness had been observed and that the foal had been found dead or extremely sick in the pasture and had died within a short time. Postmortem examination diclosed an anemia and jaundice which, according to our experience with other cases, required several days for development. It is probable that these foals were similar to those that had red blood cell counts of approximately 4,000,000/cmm. but showed few or no symptoms of ill health when protected by constant confinement in the stable. The rapidly fatal course reported by the stable attendant apparently resulted from exhaustion incident to the foal running in the pasture after its dam. Confinement and limitation of exercise until the erythrocyte count had returned to 6,000,000 to 8,000,000/cmm. appeared to be an important factor in care of the affected foal.

An analysis of 21 case histories of uncomplicated hemolytic icterus that terminated fatally showed that death occurred in 1 at twelve hours after birth, 2 at thirty hours, 1 at thirty-six hours, 4 on the third day, 8 on the fourth day, 1 on the sixth, 3 on the seventh, and 1 on the eighth. In 12 of the 21 foals illness was observed during the first twenty-four hours after birth. For 5 foals, the first observation of illness was made on the third and fourth days. Illness was not observed prior to the death of 4 foals.

The ages of mares at the time that the first jaundiced foal was produced varied from 6 to 22 years. There was much variation in the number of breeding years preceding the first foal that developed hemolytic icterus, and also in the number of pregnancies preceding the first affected foal. A summary of the breeding history of 32 mares showed that the number of breeding years prior to the first jaundiced foal varied from one to eighteen. A similar analysis for the pregnancy, at which the first jaundiced foal appeared, revealed 6 cases for the second pregnancy, 4 cases for the third, 1 for the fourth, 5 each for the fifth and sixth, 1 for the seventh, 3 for the eighth, 2 each for the ninth, tenth, and eleventh, and 1 for the twelfth. In this compilation, abortions were counted with completed pregnancies. From this, it appeared that a long series of pregnancies was not necessary for inducing isoimmunization of the mare, as 31 per cent of the first-case icteric foals resulted from the second and third pregnancies. Of the 40 mares that are now known to have produced hemolytic icterus foals, not a single case has resulted from the first pregnancy. One case of isoimmunization occurring during the first pregnancy of a mare was reported by Brion.[10] A similar report was made by Bruner *et al.*,[5] but further inquiry into the foaling history of this mare revealed that she had previously given birth to 2 dead foals. With some mares, the first icteric foal appeared after a series of two or more pregnancies by 1 stallion. In contrast to this, approximately half of the naturally immunized mares had not been pregnant twice by 1 stallion. However, this does not preclude the possibility that the mare may have borne several foals having the same red blood cell antigens.

GROSS PATHOLOGY

Except for anemia and icterus, few lesions were found regularly. The icteric discoloration of the tissues and internal organs varied with the length of time that

the foal lived. A foal dying within twelve hours of birth showed no discoloration of the serum, serous fluids, and tissues. Foals dying from twenty-four to forty-eight hours after birth showed increased intensity of the yellow staining of the tissues with advancing age, and those living seventy-two hours or longer were severely jaundiced. Cursory examination of the carcass indicated a severe anemia. In thin films or small droplets of blood, extreme clumping of the red cells was sometimes seen. This phenomenon was more commonly present in foals under 3 days old and was rarely observed in foals 5 or more days old. The color of the urine varied from light yellow to a port wine color.

Splenomegaly was observed constantly in foals dying under 48 hours of age. In these, the spleen was turgid and engorged with blood. Spleens of foals that lived four to five days or longer were approximately normal or reduced in size. They contained little blood and often were of flabby consistency. Large spleens, engorged with blood, were observed in the older foals that had recently received transfusions, probably of incompatible blood.

Because of the severe anemia, the color of the liver was lighter than normal. The liver of 1 foal that lived seven days showed gross evidence of central necrosis, and another living eight days had multiple necrotic foci throughout the liver.

Except in the very acute cases, the kidneys were swollen with increased width of the cortex. The heart was pale. Epicardial and endocardial hemorrhages were present in a few cases. There was no evidence of hypertrophy or dilatation of the heart. In most cases, the lungs showed no gross changes except icteric discoloration and small emphysematous areas.

HISTOPATHOLOGY

The tissues included in these examinations were selected from foals dying from one to eight days after birth. Postmortem examination of all foals was made within five hours after death.

Liver.—In general, the severity and extent of the degenerative and necrotic changes in the hepatic cells were augmented with increasing age of the foal at the time of death. In foals dying on the third day or later, irregular swelling, increased granularity, reduced density, and vacuolation of the cytoplasm of the hepatic cells was observed. Lysis, rhexis, and ghost forms of nuclei were seen in isolated cell groups and in association with necrotic areas. Usually, the degenerative and necrotic changes were more severe in the central portion of the lobules. Sections from a foal dying at 7 days of age showed central necrosis involving one-fourth to one-half of the area of some lobules. The liver of another foal dying at 8 days of age contained multiple necrotic foci which were dispersed throughout the liver. Erythrophagia by the Kupffer cells was observed in the livers of foals dying at 2 to 5 days of age. In the livers of foals dying at 3 days of age or later, there was dilatation of the bile canaliculi which contained an amorphous, homogenous, brownish yellow substance.

Kidneys.—Pathological changes were absent or limited to minor albuminous degeneration in foals dying within the first forty-eight hours after birth. With advancing age, the tissue changes were increased in severity and in the extent to which different parts of the nephron were affected. The pathological changes consisted of albuminous degeneration, desquamation of the tubular epithelium, and swelling, lysis, and rhexis of the nuclei. These changes were observed throughout the first anlage. Little change was seen in the collecting tubule system.

Spleen.—In foals dying at early ages and in those that received transfusions of incompatible blood shortly before death, the splenic pulp was massively engorged with red blood cells. The Malpighian bodies and trabeculae were widely separated and the architecture of the pulp was completely obscured. Spleens of foals dying at 4 days of age or later usually contained fewer red blood cells than normally present. The number of cells in the Malpighian bodies was reduced, some containing an amorphous, homogenous, hyaline-like, acidophilic substance.

Lungs.—Small emphysematous areas resulting from rupture of a few alveoli were observed. In some cases there was edematous separation of the interlobular spaces. A clear, cell-free transudate was observed in the alveoli of a few lungs.

Heart.—Very little change was found in the cardiac muscle. In foals dying at several days of age, some cardiac muscle fibers showed loss of cross striations and granular swelling of the cytoplasm.

DISCUSSION

The early appearance of clinical anemia and associated low red blood cell counts, 2,200,000 to 6,000,000/cmm., within twelve to sixteen hours after birth of the foals of naturally immunized mares, indicated rapid absorption of the colostrum antibody from the intestinal tract and an early disappear-

ance of erythrocytes from the circulating blood. Although intravascular hemolysis of circulating erythrocytes occurred in some foals, an equally severe anemia was ultimately reached in others in the absence of of discernible hemoglobinemia and hemoglobinuria. The rapid fall of erythrocyte count, in the absence of hemoglobinemia and hemoglobinuria, may be explained in part by pathological changes observed in foals dying within forty-eight hours after birth.

In these foals, the spleens uniformly showed a massive engorgement with blood. It appeared that possibly the first host reaction following absorption of the colostrum antibody upon the circulating erythrocytes was the separation of the antibody-coated erythrocytes from the circulation by the spleen. In some of the latter type of foals, there was a progressive decline in erythrocyte count and packed cell volume over a period of two to five days, and in others the lowest point in erythrocyte count was reached within twenty-four to thirty-six hours, after which the cell count increased slowly. In such foals, it appeared likely that the greater part of the destruction of the erythrocytes occurred in the spleen. This activity was evident in the erythrophagia and hemosiderin deposition in the reticular cells of the spleen. The Kupffer cells of the liver, however, were actively erythrophagocytic.

In general, the histopathological lesions occurring in the liver, kidneys, spleen, heart, and lungs were more severe as the age at time of death increased. The early deaths, at 1 to 3 days old, apparently resulted primarily from rapidly developing anemia, with the time not being adequate for pronounced cellular alterations to occur. It is probable that both the prolonged anemia and toxic effects of the jaundice contributed to the greater severity of lesions in the older foals.

In the treatment of foals affected with hemolytic icterus, it has been found that early and extensive exsanguination transfusion resulted in fewer deaths and more rapid clinical recovery. Foals not receiving adequate transfusion until the third day or later frequently showed a slower clinical response than those receiving treatment at an earlier age. It appears that the greater success attending early transfusion treatment may have resulted from prevention of damage to the internal organs which was most severe in foals dying on the fourth day or later. The exsanguination transfusion removes damaged erythrocytes and unabsorbed circulating antibody from the foal and also corrects the anemia. Removal of damaged red blood cells and antibody should reduce the severity of the jaundice, and correction of the anemia should prevent cellular damage resulting from inadequate gaseous exchange between the tissues and blood. Initial exsanguination transfusion of a quantity of blood adequate for raising the erythrocyte count to 7,000,000 to 8,000,000/cmm. appeared to give a more satisfactory response than a smaller exsanguination transfusion or periodic simple transfusions over a period of several days. For a severely affected foal having an erythrocyte count below 3,000,000/cmm., the anemia may be adequately corrected by infusion of 4 to 6 liters of fresh citrated blood and the removal of 3 to 5 liters of blood from the foal. A differential of 1 liter excess in the amount of blood infused has been tolerated satisfactorily.

In earlier studies[5, 6, 8] at this station, the severity of hemolytic icterus in foals was found to be related to the antibody titer of the dam serums and colostrums. Similar findings have been made in subsequent studies, but much variation has been encountered and in some cases the severity of the disease could not be correlated with the antibody titers of the mare serums and colostrums. For some mares, the serum and colostrum contained both hemagglutinative and hemolytic antibodies while in others only the hemagglutinin was demonstrable. Hemolysin with the absence of hemagglutinin has not been observed. The foals of mares whose serum had hemolysin titers of 1:100 to 1:1,000 usually have been severely affected, with hemoglobinemia and hemoglobinuria being a feature of the disease. Foals of mares having serum hemagglutinin titers of 1:16 to 1:32 or higher, but having hemolysin titers of less than 1:10, usually developed an early and severe anemia without hemoglobinuria.

However, some foals from mares having high serum titers of hemagglutinin have had mild cases of hemolytic icterus. Since it was not possible to demonstrate that the foal's erythrocytes were either more or less

susceptible to the activity of the maternal antibody than those of the sire or other horses reacting with it, the differences in severity of cases from mares having approximately the same serum and colostrum antibody titers appeared to be dependent upon the quantity of colostrum obtained by the foal. Apparently the foals of mares that produced a large volume of colostrum were more severely affected than those from mares producing small quantities of colostrum.

In the first cases studied, it appeared that foals of mares having colostrum titers of 1:4 or less were not in serious danger from hemolytic icterus. Subsequently, some foals born to mares which apparently produced large volumes of low titer colostrum, 1:4, have had severe cases of hemolytic icterus, 1 of which died on the third day after birth. The variation in the quantity of colostrum produced by mares was readily demonstrated by hand milking of known naturally immunized mares for the prevention of hemolytic icterus. For some mares, the removal of 4 to 6 pints of colostrum during a four- to eight-hour period eliminated demonstrable antibody or reduced it to insignificant levels. For other mares, it was necessary to remove 8 to 10 pints or more during a period of twelve to twenty-four hours to eliminate the antibody or reduce it to a level considered safe for the foal to nurse.

The most satisfactory index of the foal's condition may be obtained from daily erythrocyte counts. Experience in the Kentucky stables indicated that usually the foal could be expected to live if the erythrocyte count remained at 4,000,000/cmm., or higher, packed cell volume 14 or higher. Frequently, however, recovery was more rapid for foals in which the anemia was corrected by transfusion, particularly if the erythrocyte count remained static at approximately 4,000,000/cmm. for seven to nine days. Of foals whose erythrocyte counts do not fall to critical levels, a more favorable prognosis may be given for those showing an increase in erythrocyte count by the fifth day after birth. Erythrocyte counts of approximately 3,000,000 or less, packed cell volumes of 10 or less, indicate critical anemia and urgent need for transfusion.

For the known icteric foal-producing mare, all danger to the foal may be avoided if adequate precautions are taken at foaling time.[8] For first cases from mares not known to have become immunized against horse erythrocyte antigens, the foals are frequently in need of urgent treatment at the time the disease is diagnosed.

SUMMARY

The clinical features of hemolytic icterus of newborn foals and the foaling histories of mares producing the affected foals have been summarized and discussed.

The gross and histopathological findings in foals dying from the disease have been presented and their relation to treatment discussed.

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Some Blood Values of the Southwestern Burro (Equus Asinus Asinus)

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THERE HAS BEEN SOME recent interest in the response of a large domestic animal to externally applied whole body irradiation. For several reasons not germane to this discussion, the burro was selected. Before irradiation, it was necessary that some studies be made to determine the blood values of the healthy burro under the environmental conditions that they would be expected to encounter in eastern Tennessee. One hundred and eight animals from a band of 250 were selected for this study. The following material is a result of that work.

We have found no reference in the literature of the past twenty-five years to the subject in question. This is particularly surprising in view of extremely widespread distribution of the burro as a beast of burden.

ANIMALS

The animals were obtained for us by Dr. Fred F. Schmidt of Douglas, Ariz., from about that city and in the vicinity of Agua Prieta, Sonora, Republic of Mexico. After a period of quarantine and acclimatization, these animals were shipped to Oak Ridge, Tenn., in stock cars. Here they were placed for six months, before the study started, upon pasture consisting primarily of orchard grass and ladino clover. During the cold months, this food was supplemented with hay of the same combination of plants, plus a good quality of corn silage. At all times, more hay and silage were available than the animals would eat. It was felt that a remarkable gain in weight was evidence of adequacy of diet.

It had been noticed at autopsies that the animals were infested with ascarids which we were not able to distinguish from *Parascaris equorum* and that they had a strongyloidosis which was evidenced by the presence of an unidentified parasite of the genus Strongylus as well as parasitic aneurysms in the anterior and posterior mesenteric and renal arteries. These intestinal parasites were removed in large part by the administration of carbon tetrachloride (10 cc., approximately) on two occasions at sixty-day intervals. Subsequent autopsies indicated a considerable reduction in both species of parasites in the intestines. Parasitic aneurysms were not altered.

In so far as possible, we have eliminated pregnant females from our tabulations as well as animals suffering from coccidioidomycocis or other clinically evident diseases.

METHODS

Blood was drawn from the external jugular vein with a 15-gauge "California bleeding needle" and 10 cc. collected in dry test tubes containing 18 mg. of ammonium oxalate and 12 mg. of potassium oxalate. This was prepared according to the method of Wintrobe.[1] All except one of the subsequent tests were done within one hour of collection time. One test (films for differentials) was prepared within five minutes of venipuncture. Suspension stability of the blood was determined by the sedimentation test as described by Wintrobe,[2] except that only one reading was made at one hour and was not corrected. Red blood cell and white blood cell counts were done with the oxalated blood utilizing certified Thoma pipettes and Hayems solution for red cells and Turck's solution for white cells. For the enumeration of cells a Spencer bright-line improved Neubauer counting chamber was used.

TABLE 1—Distribution of Animals by Age and Sex

Sex	Age Group (Years)				
	1	2	3	4	5 or over
Male	16	10	2	10	3
Female	12	19	8	25	3
Both	28	29	10	35	6

Packed cell volume was determined as a continuation of the sedimentation test. Using Wintrobe tubes, following the reading of the sedimentation test, the tubes containing the blood were spun in a centrifuge at 1650 revolutions per minute (r.p.m.) for thirty minutes at 25.5-cm. distance. This was equivalent to

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Dr. Kimball is from the Mathematics Panel, Oak Ridge National Laboratory. Lt. Colonels Trum and Rust are from the Veterinary Corps, United States Army.

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TABLE 2—White Blood Cells—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	8,850	7,750	14,150	9,150	7,000	
		16,700	17,700	14,450	16,900	10,750	
	Mean	12,900	12,600	14,300	12,940	9,000	12,620
Female	Range	9,650	9,350	10,350	10,150	11,100	
		28,550	20,400	19,200	19,350	17,150	
	Mean	16,400	14,070	15,390	13,220	14,720	14,360
Both	Mean	14,400	13,560	15,180	13,140	14,360	13,700

TABLE 3—Analysis of Variance of White Blood Cells (Coded by Dividing by 10^2)

Source	d.f.	m.s.	F	Level
Age	4	19.89450	2.37	N.S.
Sex	1	89.29010	10.66	1%
Interaction	4	15.10150	1.80	N.S.
Error	98	8.37893	...	...

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

TABLE 4—Red Blood Cells—Extremes and Means (Coded by Dividing by 10^6)

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	5.65	5.93	5.92	6.28	6.97	
		8.33	7.42	6.40	8.58	7.66	
	Mean	6.71	6.63	6.16	7.55	7.32	6.91
Female	Range	5.33	5.45	4.95	5.38	6.77	
		7.87	10.27	8.38	8.42	8.07	
	Mean	6.63	7.34	7.41	7.00	7.45	7.10
Both	Mean	6.68	7.10	7.16	7.16	7.37	7.03

TABLE 5—Analysis of Variance of Red Blood Cells (Coded by Dividing by 10^6)

Source	d.f.	m.s.	F	Level
Age	4	2.521000	3.5900	1%
Sex	1	0.012150	0.0173	N.S.
Interaction	4	1.948500	2.7800	5%
Error	98	0.701600		

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

TABLE 6—Hemoglobin—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	9.90	9.30	9.60	10.70	11.20	
		14.20	12.10	10.80	15.50	13.40	
	Mean	11.34	11.06	10.20	12.67	12.37	11.61
Female	Range	8.60	9.20	8.00	10.00	12.80	
		12.70	16.70	15.50	13.90	15.10	
	Mean	11.13	12.50	12.66	11.92	13.77	12.11
Both	Mean	11.25	12.00	12.17	12.13	13.07	11.93

TABLE 7—Analysis of Variance of Hemoglobin

Source	d.f.	m.s.	F	Level
Age	4	6.54300	3.63	1%
Sex	1	10.93750	6.07	5%
Interaction	4	6.83680	3.79	1%
Error	98	1.80312	...	...

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance.

TABLE 8—Sedimentation Rate—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	47.00	39.00	61.00	21.00	49.00	
		63.00	64.00	61.00	61.00	57.00	
	Mean	57.25	57.00	61.00	46.50	52.00	54.37
Female	Range	12.00	4.00	17.00	13.00	31.00	
		67.00	65.00	68.00	62.00	50.00	
	Mean	53.08	41.84	42.13	51.92	39.67	47.55
Both	Mean	55.46	47.07	45.90	50.37	45.83	50.14

TABLE 9—Analysis of Variance of Sedimentation Rate

Source	d.f.	m.s.	F	Level
Age	4	198.993	1.240	N.S.
Sex	1	1175.698	7.310	1%
Interaction	4	475.194	2.840	5%
Error	98	160.881	....	...

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

TABLE 10—Hematocrit (Packed Cell Volume)—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	31.00	31.00	31.00	34.00	35.00	
		43.00	39.00	33.00	45.00	41.50	
	Mean	35.13	34.60	32.00	38.50	38.67	35.93
Female	Range	29.00	28.50	26.00	30.00	41.00	
		39.50	51.00	44.00	41.00	43.00	
	Mean	35.33	38.16	38.75	36.76	42.00	37.37
Both	Mean	35.21	36.93	37.40	37.26	40.33	36.82

TABLE 11—Analysis of Variance of Hematocrit

Source	d.f.	m.s.	F	Level
Age	4	44.1305	2.72	5%
Sex	1	84.6700	5.21	5%
Interaction	4	40.2938	2.48	5%
Error	98	16.2507	...	...

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance.

TABLE 12—Heterophiles (Segmented Only, Expressed as a Percentage of the Total White Cell Count)—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	26.00	32.00	33.00	26.50	39.00	
		52.50	65.50	55.00	52.50	65.00	
	Mean	39.63	43.60	46.50	45.50	50.00	43.12
Female	Range	28.00	28.00	42.00	33.50	36.00	
		64.00	58.00	51.00	55.50	48.00	
	Mean	48.00	43.21	45.00	39.72	40.33	42.85
Both	Mean	43.21	43.34	45.30	41.37	45.17	42.95

TABLE 13—Analysis of Variance of Heterophiles

Source	d.f.	m.s.	F	Level
Age	4	18.6186	0.278	N.S.
Sex	1	46.7800	0.700	N.S.
Interaction	4	215.8512	3.230	5%
Error	98	66.8369	....	...

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

TABLE 14—Eosinophiles (Expressed as a Percentage of the Total White Cell Count)—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	1.50	0.00	9.00	4.00	5.00	
		18.00	14.00	11.50	12.50	8.50	
	Mean	7.88	7.60	10.50	6.40	7.00	7.51
Female	Range	0.50	1.00	2.00	2.50	11.00	
		12.50	14.00	12.50	26.00	23.00	
	Mean	6.67	7.58	8.25	8.32	15.33	8.12
Both	Mean	7.36	7.59	8.70	7.77	11.17	7.89

TABLE 15—Analysis of Variance of Eosinophiles

Source	d.f.	m.s.	F	Level
Age	4	20.3076	1.05	N.S.
Sex	1	8.5094	0.44	N.S.
Interaction	4	35.0100	1.81	N.S.
Error	98	19.3465	...	....

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

TABLE 16—Monocytes (Expressed as a Percentage of the Total White Cell Count)—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	2.00	1.00	2.50	3.00	3.50	
		8.50	11.50	6.00	13.50	8.00	
	Mean	4.94	5.60	4.00	7.80	6.33	5.85
Female	Range	4.00	0.00	1.00	1.50	3.50	
		8.00	9.50	9.00	11.50	7.00	
	Mean	5.83	3.68	4.63	7.12	5.00	5.52
Both	Mean	5.32	4.34	4.50	7.31	5.67	5.65

TABLE 17—Analysis of Variance of Monocytes

Source	d.f.	m.s.	F	Level
Age	4	41.5553	6.66	1%
Sex	1	6.4181	1.03	N.S.
Interaction	4	7.4392	1.19	N.S.
Error	98	6.2438	...	....

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

TABLE 18—Lymphocytes (Expressed as a Percentage of the Total White Cell Count)—Extremes and Means

Sex		Age Groups (Years) 1	2	3	4	5 or over	Mean
Male	Range	32.50	25.50	37.00	28.50	18.50	
		64.00	56.00	39.50	45.50	47.50	
	Mean	45.63	42.80	38.50	39.70	35.33	42.39
Female	Range	24.00	29.00	30.00	35.00	34.00	
		53.00	57.50	59.00	54.50	44.00	
	Mean	37.33	44.26	41.38	44.04	38.00	42.31
Both	Mean	42.07	43.76	40.80	42.80	36.67	42.34

770 G.* Preliminary tests have indicated that no measurable change in the final reading could be anticipated following a longer time in the centrifuge.

Hemoglobin was determined by a colorimetric method in a Klett-Summerson colorimeter. Iron was measured after being detached from the hemoglobin molecule by the method of Dupray[3] as modified by Wong[4] and Hanzal.[5] From the iron values obtained, hemoglobin was estimated on the basis of 3.4 mg. of iron in each 1 Gm. of hemoglobin. The findings are expressed as grams of hemoglobin in 100 ml. of blood.

As mentioned before, films for differential estimation of leukocytes were prepared immediately after blood-letting. These films were stained by Wright's method using the coverslip technique for film preparation. We had been warned of the bizarre-appearing cells that are present on films made of oxalated human blood that had been held unduly long. We had occasion to observe this phenomenon in burro blood. Except for the eosinophils, the white cells of the burro are not essentially different from those of other mammals that we have had occasion to observe. The eosinophil, in common with other members of the genus Equus, have large ruby red granules that often obscure the nucleus.

TABLE 19—Analysis of Variance of Lymphocytes

Source	d.f.	m.s.	F	Level
Age	4	71.5080	1.0800	N.S.
Sex	1	2.5298	0.0381	N.S.
Interaction	4	160.5816	2.4200	N.S.
Error	98	66.4061	.....	....

d.f. = degree of freedom (n-1); m.s. = the mean significant or variance (s^2); F = variance ratio; Level = the level of significance; N.S. = no significant difference.

RESULTS AND STATISTICAL ANALYSIS

Basophiles.—These were not always found. Most commonly they were 0.5 per cent of the total white cell population, though on one occasion they rose to 2.0 per cent. There were no sex or age differences.

Band Cells.—These were much like the basophiles except for being slightly higher. They range from 0.0 to 3.0 per cent. There were no sex or age differences.

Reticulocytes.—We routinely stained our blood films with brilliant creysl blue. Reticulocytes were entirely absent from the peripheral blood. This suggests one of three things, either the vital stain used was not capable of staining reticulocytes, our method was unsatisfactory, or that reticulocytes do not occur in the peripheral blood of the burro. We suspect the latter since we did have success in staining reticulocytes in the bone marrow.

*Gravitational Constant (32.16 ft./sec./sec.).

TABLE 20—Results of Blood Tests of Healthy Burros

White Blood Cells—Males, 12,620; females, 14,360; both, 13.700. There was a significant difference between sexes. There was some indication that the range was greater in the young animals than in the mature.
Red Blood Cells—Males, 6,910,000; females, 7,100,000; both, 7,030,000. There was a significant difference when examined by age. A moderately significant difference in the interaction is evidence that the difference in age groups was not the same for males as females.
Hemoglobin—Males, 11.61 Gms. for each 100 ml. of blood; females, 12.11 Gms. for each 100 ml. of blood; both, 11.93 Gms. for each 100 ml. of blood. There was a significant difference between age groups and moderate degree of significance when compared by sex. The interaction suggested a difference in pattern in both age and sex.
Sedimentation Rate—Males, 54.37; females, 47.55; both, 50.14. There was no significance between ages but there was a significant difference between sexes. A moderately significant difference existed in the pattern by sexes.
Hematocrit—Males, 35.93; females, 37.37; both, 36.82. There was only a moderate degree of significance in the difference between sexes, age and interaction.
Heterophiles—Males, 43.12; females, 42.85; both, 42.95. No significant difference exists by sex or age, though there was a moderate interaction variance.
Eosinophiles—Males, 7.51; females, 8.12; both, 7.98. There was no significant difference in age, sex or interaction.
Monocytes—Males, 5.85; females, 5.52; both, 5.65. There was a significant difference between ages but none in sex and interaction.
Lymphocytes—Males, 42.39; females, 42.31; both, 42.34. There was no significant difference between ages, sexes or in the interaction.
Basophiles—Range 0.5 to 2.0%. There was no sex or age difference. *Band Cells*—Range 0.0 to 3.0%. There was no sex or age difference. *Reticulocytes*—None were found.

All data are mean values.

SUMMARY

A summary and statistical analysis is presented of the various blood cytology values found in 108 burros ranging in age from 1 to 5 years, or over. Both sexes are represented.

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Normal Variances in Horse Blood Due to Breed, Age, Lactation, Pregnancy, and Altitude

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IN ORDER TO understand some effects of high altitude on the horse, a clinical survey of the blood cytology of 265 remount horses was made in Cochabamba, Bolivia, during 1949-1951. The results are presented here as a contribution to the general knowledge of the hematology of the horse.

HISTORY OF SUBJECTS

There were 265 horses examined in this work. Geldings, stallions, and mares of ages varying from 2 days to adulthood were included. The majority of the animals were of mixed breeding although some Percherons and Thoroughbreds are included. The purebred animals were fed grain and alfalfa hay in the stables; the mixed breeds grazed in green alfalfa during the day and had corn fodder in their corrals at night. A routine program of parasite control consisting of periodic administration of vermifuge (phenothiazine and carbon bisulfide) and pasture rotation was practiced on the farm. None of the animals was considered to be heavily parasitized. Twenty-four mares were thin, having been imported from Argentina about two months before samples of blood were drawn. The yearlings were also thin. At the time of bleeding, none of the animals was sick. The foals were from 2 days to 4 months old. The mares were grouped as barren and dry, barren and lactating, pregnant and dry, and pregnant and lactating. They were living at an altitude of 2,550 meters (8,375 ft.) above sea level.

METHODS

The blood was drawn from the left jugular vein through a 15-gauge bleeding needle. It was taken between 8 and 10 a.m. Animals were handled as quietly as possible. The purebred animals stood quietly. Some of the mixed breeds, particularly the young foals, were excited when caught. No restraint other than a halter was used.

About half of the time, the blood counts and differentials were made from blood taken directly from the bleeding needle. Otherwise, all tests were done within an hour from 10 ml. of blood drawn into a test tube into which a solution of sodium oxalate had been placed and the water evaporated, leaving approximately 20 mg. of sodium oxalate. If clotting occurred, the sample was not used.

For differential counts of white blood cells, the blood films were made from freshly drawn blood using the spreader slide technique and were stained with Wright's stain within a few hours. Not less than 100 cells were counted from each sample and several hundred were counted when distribution seemed unusual.

Hemoglobin was determined by the Sahli method. The calibration of tubes and pipettes were verified. Fluid color standard was used.

Red blood cells were diluted with Hayems solution (1:200) in certified pipettes and counted in a Neubauer counting chamber. Single counts were made on most samples and verified if considered unusual.

Blood was diluted in certified white cell pipette (1:20) with 1 per cent acetic acid and counted in a Neubauer counting chamber. One count was made on most samples. Unusual samples were verified by a new dilution.

Cell volume was determined in Wintrobe hematocrit tubes centrifuged at 3,000 revolutions per minute for forty-five minutes.

The arithmetical mean (average), standard deviation, and standard error were determined for each group. Significance was attributed only at the level of $P = 0.05$ or more than twice the standard error of the mean. Polygons (fig. 1) were made of distribution frequencies of major (most numerical) classes for the two values, hemoglobin and erythrocyte count (MCH). There seems to be sufficient symmetry to permit analysis of statistical data.

RESULTS

The results of the analyses are shown in table 1. The various blood values of the different classes of horses examined are given. These different classifications can not properly be compared, one with another, without introducing factors which might vitiate conclusions. For example, the erythrocyte count of the blood of the foals ($10.75 \pm .40$ millions per cmm.) can not properly be compared with that of the adult Thoroughbreds ($9.37 \pm .32$) or adult

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TABLE I—Analyses of the Blood Values of the Different Classes of Horses

Classification and number of Animals	Statistics	Hemoglobin Grams/100 Ml	Erythrocytes 10^6/cmm.	Leukocytes 10^3/cmm.	Eosinophils %	Monocytes %	Neutrophils %	Lymphocytes %
Yearlings	Mean and s.e.	11.27 ± .18	8.13 ± .18	10.84 ± .19	4.8 ± .87	1.8 ± .26	49.8 ± 1.83	43.6 ± 1.78
(53)	Range	0.8 - 15.9	5.80 - 10.65	5.0 - 15.0	0 - 15	0 - 6	28 - 71	28 - 66
	Standard Deviation and s.e.	1.30 ± .12	1.27 ± .12	1.40 ± .13	5.0 ± .61	1.4 ± .18	10.50 ± 1.30	10.24 ± 1.26
Foals	Mean and s.e.	13.42 ± .27	10.75 ± .40	10.25 ± .56	3.5 ± .52	2.0 ± .45	45.0 ± 2.62	49.5 ± 2.9
(28)	Range	9.8 - 15.9	7.40 - 16.70	5.70 - 15.45	0 - 15	0 - 8	20 - 71	23 - 76
	Standard Deviation and s.e.	1.43 ± .19	2.19 ± .30	2.99 ± .39	2.67 ± .36	2.32 ± .31	13.37 ± 1.85	14.83 ± 2.0
Percherons (11)	Mean and s.e.	11.67 ± .51	7.39 ± .40	8.01 ± .56	4.7 ± 1.2	1.5 ± .53	52.2 ± 3.6	41.1 ± 4.0
4 males	Range	10.1 - 14.5	5.70 - 9.55	6.00 - 10.50	2 - 13	0 - 5	36 - 74	20 - 59
7 females (Barren)	Standard Deviation and s.e.	1.27 ± .36	1.28 ± .28	1.78 ± .40	3.87 ± .86	1.67 ± .37	12.20 ± 2.7	13.45 ± 3.0
Riding Horse Geldings (12)	Mean and s.e.	11.83 ± .39	8.60 ± .53	8.03 ± .41	8 ± 1.9	1 ± .36	45 ± 3.2	46 ± 2.6
	Range	10.2 - 14.5	5.40 - 11.70	5.50 - 10.85	1 - 14	0 - 3	20 - 61	30 - 61
	Standard Deviation and s.e.	1.31 ± .28	1.87 ± .38	1.44 ± .29	6.6 ± 1.3	1.04 ± .23	11.22 ± 2.3	9.21 ± 2.0
Thoroughbreds 9 females 14 males (23)	Mean and s.e.	12.7 ± .28	9.37 ± .32	7.94 ± .41	8.8 ± 1.61	2.2 ± .68	50.5 ± 3.7	38.5 ± 3.6
	Range	10.2 - 14.8	6.50 - 11.60	5.80 - 10.85	1 - 18	0 - 6	32 - 69	10 - 57
	Standard Deviation and s.e.	1.36 ± .20	1.58 ± .23	1.65 ± .29	5.40 ± 1.17	2.25 ± .48	12.4 ± 2.6	12.24 ± 2.6
Pregnant Not milking (31)	Mean and s.e.	12.6 ± .26	8.14 ± .42	8.85 ± .36	5.8 ± .50	1.8 ± .24	45.6 ± 2.17	46.8 ± 1.34
	Range	9.8 - 15.7	5.44 - 10.85	5.50 - 12.35	1 - 14	0 - 4	24 - 62	31 - 74
	Standard Deviation and s.e.	1.44 ± .18	2.34 ± .30	1.98 ± .25	3.52 ± .40	1.3 ± .16	11.87 ± 1.53	10.39 ± 1.89
Pregnant Milking (15)	Mean and s.e.	12.0 ± .36	8.18 ± .30	8.38 ± .36	7.5 ± 1.1	1.5 ± .29	45.0 ± 2.98	46.0 ± 3.18
	Range	9.5 - 14.1	6.0 - 11.5	6.45 - 11.2	2 - 14	0 - 3	30 - 63	26 - 63
	Standard Deviation and s.e.	1.43 ± .26	1.18 ± .21	1.42 ± .26	4.12 ± .77	1.09 ± .2	11.13 ± 2.10	11.95 ± 2.26
Barren Not Milking (63)	Mean and s.e.	12.3 ± .20	8.89 ± .14	9.69 ± .31	4.3 ± .41	1.6 ± .23	48.1 ± 1.49	46.0 ± 1.06
	Range	9.8 - 14.8	4.58 - 14.35	5.60 - 14.30	0 - 15	0 - 8	23 - 65	20 - 67
	Standard Deviation and s.e.	1.60 ± .14	1.13 ± .10	2.53 ± .22	3.27 ± .29	1.85 ± .16	11.83 ± 1.05	8.48 ± .75
Barren Milking (29)	Mean and s.e.	12.08 ± .28	8.15 ± .29	7.55 ± .25	6.8 ± .88	1.6 ± .50	44.42 ± 1.89	47.2 ± 2.16
	Range	7.3 - 14.5	5.45 - 11.20	5.10 - 10.80	1 - 19	0 - 6	22 - 57	30 - 66
	Standard Deviation and s.e.	1.51 ± .2	1.55 ± .2	1.32 ± .17	4.69 ± .62	2.73 ± .40	10.04 ± 1.34	11.44 ± 1.53
Lactating (44)	Mean and s.e.	12.0 ± .31	8.16 ± .29	7.83 ± .28	7.0 ± .92	1.7 ± .43	44.6 ± 2.15	46.7 ± 2.50
	Range	7.3 - 14.5	5.45 - 11.5	5.10 - 11.2	1 - 19	0 - 6	22 - 63	26 - 66
	Standard Deviation and s. e.	1.45 ± .2	1.33 ± .2	1.35 ± .2	4.5 ± .67	2.18 ± .2	11.84 ± 1.21	9.12 ± 1.16
Non Lactating (94)	Mean and s.e.	12.6 ± .2	8.64 ± .27	9.41 ± .33	4.8 ± .44	1.6 ± .23	47.3 ± 1.72	46.3 ± 1.15
	Range	9.8 - 15.7	4.58 - 14.35	5.5 - 14.3	0 - 15	0 - 8	23 - 65	20 - 70
	Standard Deviation and s.e.	1.48 ± .15	1.53 ± .17	2.34 ± .02	3.3 ± .33	1.88 ± .16	10.4 ± 1.59	11.6 ± 1.77

Percherons (7.39 ± .40) for an age comparison without introducing a breed difference, which in this case is significant. It would be proper to compare the foals with the yearlings and brood mares, all of which are of the same mixed breeding. Because of improper comparisons some apparent differences are illusory.

The following are statistically significant differences:

1) Foals were higher in hemoglobin (13.42 ± .27 Gm. per ml. of blood) than mares (12.4 ± .25) or yearling (11.27 ± .18). The yearlings were lower in hemoglobin than either the foals or mares.

2) The Thoroughbreds had more red cells (9.37 ± .32 million per cmm.) than the Percherons (7.39 ± .40). The foals (10.75 ± .40) also had higher erythrocyte count than the mares (8.48 ± .27) or yearlings (8.13 ± .18).

3) Both foals (10.25 ± .56 thousand per cmm.) and yearlings (10.84 ± .19) had more white blood cells than the mares (8.88 ± .31). An interesting difference between lactating and nonlactating mares was observed. The lactating mares had fewer (7.83 ± .28) cells than the nonlactating (9.41 ± .33). The interaction of this difference in pregnancy and in bareness was substantiating evidence of a significant difference.

4) Eosinophils were found more often in Thoroughbred blood (8.8 ± 1.61% of white cells) than in the blood of Percherons (4.7 ± 1.20%). Lactating mares

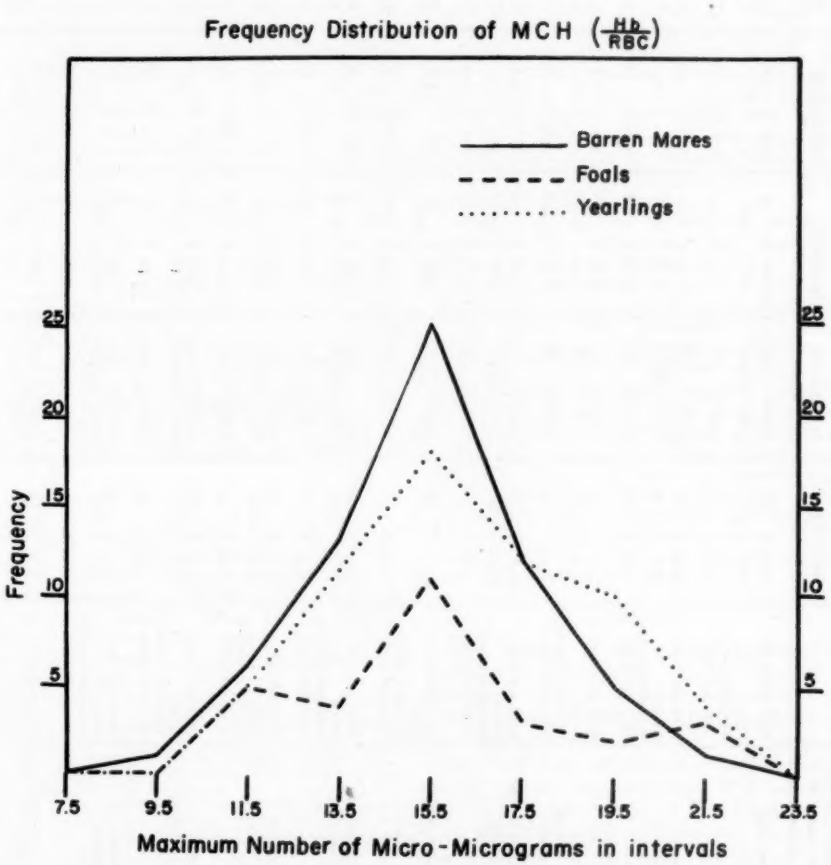

Fig. 1—Distribution frequencies of major classes hemoglobin.

had more ($7.0 \pm .92\%$) than nonlactating ($4.8 \pm .44\%$).

Two determined values are not included in table 1. There were so few basophils found that they are not computed statistically. They occurred in the order of .10 per cent of white cells. The packed cell volume of blood for the whole group averaged 40 per cent with a range of 25 to 55 per cent. The packed cell volume of blood for yearlings only was 33 per cent with a range of 25 to 50 per cent.

Discussion

It is now apparent that divergent values for formed constituents in the blood of the horse are to be expected. It is the inherent differences between horses and in their environment that cause these variations. A general pattern is emerging which allows us to evaluate specific data.

Breed differences in the blood cytology of the horse have been mentioned by other authors. MacLeod *et al.*[12] have noted that Thoroughbreds have more red blood cells per milliliter of blood than "cold-blooded" breeds. Neser[13] found a similar difference. Romijn [14] reported a significant difference in both red blood cells and hemoglobin between cold-blooded horses and "warmblooded" horses. However, Romijin's warmblooded animals were not Thoroughbreds and resembled in breed characteristics the American Standard Bred considered as cold-blooded by MacLeod. When the work of Hansen *et al.*[6-10] on Thoroughbreds is compared with that of Stewart and Holman[15] reporting on Clydesdales the same difference is noted. We found that the Thoroughbreds had a significantly higher erythrocyte count than the Percheron but no significant difference in the amount of hemoglobin.

The possibility of sex differences in blood values was considered. Hansen *et al.*[7] found the sex difference in weanlings to be statistically insignificant. Romijn[14] found a small but not significant difference of red blood cells between sexes in mature horses and quoted Wirth as finding the same, but said that Vaulent[17] considered the difference significant. Romijn wrote that he and Van Den Berg [1] found no significant difference in hemoglobin between the sexes. Only among the yearlings and foals did we have sufficient numbers to be of value and no difference was noted. It seems very probable that there is no significant difference in blood cytology due to sex alone.

We found that age made a significant difference in red blood cell count, hemoglobin, and white cells. In general, the foals were higher than adults and the yearlings lower, except that both yearlings and foals were higher than adults (mares) in white blood cells. This also is what is found in the human being, according to Wintrobe,[18] if it is considered that the foal is an infant and the yearling is an adolescent. We could not find this exact pattern in MacLeod's[12] nor in Hansen's[6-10] work, although in both cases there was a significant increase of leucocytes in the young over the adults. Todd *et al.*[16] mentioned the fact that they did not observe an immediate decrease in total erythrocytes after birth in the foal. We have observed this phenomenon several times. The highest count recorded was a foal immediately after birth (16.7 million) which had dropped to 13.0 million in thirty-six hours. The others were not quite as high but the depression was of the same magnitude. Incompleted studies on blood volume made by us seem to indicate that this extremely high erythrocyte counts of the newborn may be a manifestation of low blood plasma volume which rapidly corrects itself. Romijn[14] concluded that age did not influence the hemoglobin content of the blood, however, he thought that younger warmblooded mares had a higher packed cell volume and noted that Van Den Berg[1] reported a like difference among 250 Australian horses. However, in this case, both classes were adult animals, the young being under 10 years and the old over 10 years of age.

Before leaving the influence of age, it might be pointed out that of all horses the young foal is the most difficult to bleed without frightening it. A truly young foal will become frightened when held firmly, however gently. Neser[13] stated that an increased pulse rate caused by excitement can raise the red blood cells in jugular blood by nearly a million cells per cubic millimeter. Hansen *et al.*[6] demonstrated that it might be increased even as much as 4 million in a mare. Therefore, the blood count of young timid animals may be raised by this excitement alone.

It will be recalled that the yearlings and

a few mares, subjects of this report, were thin. Physical fitness might conceivably be a cause of a change in blood constituents, but MacLeod[9] found that in well-nourished animals a high degree of fitness did not alter the erythrocyte count. Neser[13] endeavored to show that the blood count of the horse depended to a large extent on the physical condition — stalled horses ranged from 3.9 to 7.0 million red blood cells per cubic millimeter, horses doing fast work from 7.4 to 11.5, and race horses from 9.0 to 11.0. It is clear that this has not demonstrated the relationship of blood counts to physical fitness because a type and breed factor has been introduced which could account for the differences shown. It does not follow, *a priori*, that poor condition can not affect the blood value, but it has not been demonstrated. One is apt to associate parasitism with thin horses. However, Hansen *et al.*[7] has reported that light to moderate infestations of parasites did not significantly alter erythrocyte counts.

The cellular content of the nonlactating mare's blood is higher than that of the lactating mare. Hansen *et al.*[10] also noted this.

There was no significant difference found in our animals when the blood cytology of pregnant and barren mares were compared. Also, when the blood values of the pregnant and barren mares, reported by Hansen *et al.*[6, 9] were compared there was no differences noted. However, MacLeod *et al.*[12] found a lower count in the barren mares.

An interesting observation was made in a few pregnant mares listed in table 2.

TABLE 2—Red Blood Cell Count of Pregnant Mares

Identity of mare (no.)	Red blood cells per ml., count before foaling	Red blood cells count per ml., after foaling
30	7,369,000	8,000,000 (1 week)
191	5,600,000	10,000,000 (2 weeks)
2	8,400,000	12,500,000 (1 day)
108	8,080,000	11,250,000 (3 days)
Average	7,382,000	10,440,000

Too few mares have been observed to state more than that there appears to be an anemia in late pregnancy, followed by a polycythemia immediately following pregnancy. This physiological change is noted in women. Dieckmann and Wegner[3] suggested that it can be explained by an increase of 25 per cent plasma volume and 23 per cent blood volume in the woman at term. This imbalance is rectified by a loss of fluids.

Foals and yearlings had more white blood cells than the older animals (mares). Thoroughbreds had more eosinophils than the Percherons and the lactating mares had more than the nonlactating. The range is wide and the standard deviation large on the differential counts of leukocytes. These results compare well with those reported by Hansen,[6,9] Todd,[16] MacLeod[12], Stewart,[15] and Dukes.[4]

TABLE 3—Mean Corpuscular Hemoglobin

Classification of horse	Mean corpuscular hemoglobin (Hb.) / (r.b.c.) in micro micrograms: This paper	Results of authors: Hansen (Todd)	MacLeod
Foals	12.4	10.3	12.0
Weanlings	....	10.6	10.9
Yearlings	13.8	....	11.7
Pregnant	15.0	14.1	13.7
Barren	13.7	13.9	15.2
Lactating	14.7	14.2	....
Nonlactating	14.5	14.0	....
Draft horses	16.1	....	15.9
Riding horses (geldings)	13.7	....	14.2
Thoroughbreds	13.5*	13.74*	13.3**

*Stallions and barren mares. **Stallions, geldings, and barrens mares.

Table 3 was prepared from our data and figures in the reports of Hansen *et al.*,[6,10] Todd,[16] and MacLeod.[12] The mean corpuscular hemoglobin is used because by this we may compare at a glance the ratio of two separately computed values. For the most part, there is a nice agreement between the MCH values. Many of the contradictory results in previous studies mentioned by Romijn[14] when he compared his results with those of Kuhl,[11] Van Den Berg,[1] Wirth,[19] Vaulant,[17] and Berg[2] can be explained as normal variations of different classes of horses.

An increase in erythrocytes due to altitude, which was noted by Esponoza[5] in the human population of Cochabamba, Bolivia, was not established in the horse. When we compared the blood values of Clydesdales given by Stewart and Holman[15] with the blood values of our Percherons, we found an increase in red blood cells. A similar increase due to altitude may be inferred by a comparison of the normal, as

given by Dukes,[4] with our averages. However, when the Thoroughbreds reported here were compared with the results of MacLeod[12] and Hansen *et al.*[6-8] this difference was not found. There is, therefore, no reliable evidence for or against an increase of the red blood cells in horses due to high altitude alone.

SUMMARY

The cell counts and hemoglobin content of the blood of 265 Bolivian remount horses are given. A breed and age difference in cell counts is indicated as well as a difference during lactation. The sex of the animal did not seem to effect the blood values significantly. Pregnancy seems to result in a near term anemia and a postpartum polycythemia. Neonatal polycythemia was noted. It is demonstrated that the practice of comparing absolute numbers taken from blood values from incomparable samples will lead to false and unwarranted deductions.

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The Effect of Some N-Heterocyclic Compounds on the Developmental Stages of Horse Strongyles

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LEVINE[13] (1949) REPORTED that 0.01 per cent (0.00038 M.) nicotine sulfate killed 99 per cent of the developmental stages of horse strongyles in feces. This pyridine-pyrrolidine compound is a standard anthelmintic for *Ascaridia galli* in poultry. Because of its activity against nematodes and because other N-heterocyclic compounds have been found to be toxic for arthropods and other organisms, it was felt worthwhile to screen various pyridine, piperidine and other N-heterocyclic compounds against horse strongyle larvae.

Parnell[16, 17] reported that 0.25 per cent of pyridine killed horse strongyle larvae in feces, but that it required 2.9 per cent of nicotine sulfate to do so. Cameron and Parnell[2] found that 0.15 per cent of oxyquinoline sulfate killed horse strongyle larvae in feces. Levine[15] reported that several N-iodides of pyridine, piperidine, and quinoline were very active against horse strongyle larvae, but that this activity was probably due to the iodide ion rather than to the basic rings. Vioform (5-chloro-7-iodo-8-hydroxyquinoline) was inactive.

Baldwin[1] studied the effect of a number of N-heterocyclic compounds on the contractility of anterior sections of *Ascaris lumbricoides*. Among ten thiazole derivatives, only two were active. The most effective of nine pyridine compounds was 4-benzylpyridine, which caused paralysis in a concentration of 1 : 10,000. Pyridine, 2-methylpyridine, and 2-aminopyridine caused paralysis at 1 : 5000, 2-chloropyridine and arecoline did so at a concentration of 1 : 1000, while 1-methyl-2-pyridine, sulfapyridine, and nikethamide failed to cause paralysis at that concentration. Baldwin found that 2,2′-dipyridyl, which caused paralysis at a concentration of 1 : 100,000, was by far the most active of the dipyridyls he studied. He found further that 2,2′,2″-tripyridyl caused paralysis at 1 : 50,000, and that 4,5-phenanthroline did so at 1 : 100,000 while 1,5-phenanthroline did so only at 1 : 1000 and 1,8-phenanthroline was inactive.

Hewitt *et al.*[4-7] studied the effects of a large number of piperazine derivatives against *Litomosoides carinii* in the cotton rat and *Dirofilaria immitis* in the dog. Of these 1-diethylcarbamyl-4-methylpiperazine (= caricide, = hetrazan) was selected as the most promising. It has been used successfully against *Wuchereria bancrofti* and other filariids in man, and an extensive literature has been built up on its use in this host. It is a standard treatment for *Dirofilaria immitis* in the dog. In addition, Hewitt *et al.*[7] and Colglazier and Enzie[3] found that it was effective against ascarids but not against hookworms, whipworms, or tapeworms in the dog. Riedel[21] reported that it was effective against *Ascaridia galli* in the chicken, and Werner[26] used it successfully in a dog with Strongyloides infection. Todd, Hansen, Kelley and Wyant[23] found that caricide was ineffective against *Strongylus vulgaris* fourth stage larvae in the horse.

Kartsonis and Austin[8] reported that N-methyl tetrahydro methyl nicotinate-*p*-carboxyphenyl stibonic acid (= anthelin) was effective against *Dipylidium caninum*, Taenia sp., *Toxocara canis*, and *Toxascaris leonina* in dogs.

Tests of a large number of N-heterocyclic compounds, and particularly of quinoline and pyridine compounds, against several species of Plasmodium have been summarized by Wiselogle.[27] Many quinoline compounds are active antimalarials, but the pyridine compounds have little activity.

Studies on the effects of pyridine, dipyridyl, piperidine, and other N-heterocyclic compounds on arthropods have been reported by Richardson and Smith,[20] Smith, Richardson and Shepard,[22] Richardson and Shepard,[19] Richardson, Craig and Hansberry,[18] King and Frear,[9-11] and King, Frear and Dills.[12] Their findings will be discussed below.

METHOD

The technique used in the present study has been described by Levine.[13] Briefly, it consisted of mixing the compound to be tested with talcum powder at ten times the desired final concentration, and then mixing 0.5 Gm. of this mixture with 4.5 Gm. of fresh horse feces containing strongyle eggs. The final mixture was suspended in a cheesecloth bag in a closed, 2-ounce, wide-mouth glass jar. The jar was placed in a dark cupboard for a week and then examined for larvae. The condensation water in the jar was examined, and the larvae in the

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feces were concentrated with a Baermann apparatus for examination.

Concentrations of 0.01, 0.005, 0.0025, 0.001, 0.0005, and 0.00025 M. were employed for each compound. Untreated controls were set up at the same time. The presence of larvae in a jar was taken to indicate that the compound was ineffective. In a few exceptional cases, a very few larvae were found with the Baermann apparatus. Since these were much less than 1 per cent of the numbers found in the controls, the compound was considered effective in these cases.

The feces used in this study were obtained from several horses on a single farm. Of 2,200 larvae identified from animals on this farm, 99.4 per cent were those of small strongyles, 0.55 per cent of *S. vulgaris* and 0.05 per cent of *Strongylus edentatus*.

RESULTS

The lowest lethal concentrations (in moles) are listed below.

Pyridine and its derivatives

pyridine	0.01 inactive
3-methylpyridine*	0.01 inactive
2,6-dimethylpyridine*	0.01 inactive
2-hexylpyridine*	0.00025
n-dodecyl bromides of mixed methyl pyridines*	0.01 inactive
2-vinyl-6-methylpyridine*	0.005
2-vinylpyridine*	0.005
2-aminopyridine***	0.01
2-(1′-ethanol) pyridine*	0.01 inactive
2-(2′-ethanol)-6-methylpyridine*	0.01 inactive
2,6-di (2′-ethanol) pyridine*	0.01 inactive
nicotinic acid**	0.01 inactive
isonicotinic acid*	0.01 inactive
methylpicolinate*	0.01 inactive
methylisonicotinate*	0.01 inactive
dipicolinic acid*	0.01 inactive
nicotinamide**	0.01 inactive
chlorinated 2-methylpyridine (*c.* 4 Cl)*	0.01 inactive
chlorinated 2-methylpyridine (*c.* 5 Cl)*	0.01 inactive
2-trichloromethyl-3,4,5-trichloropyridine*	0.01 inactive
chlorinated 3-methylpyridine (*c.* 4 Cl)*	0.0025
chlorinated 4-methylpyridine (*c.* 5 Cl)*	0.01
chlorinated 2,4-dimethylpyridine (*c.* 5 Cl)*	0.01 inactive
chlorinated 2,4-dimethylpyridine (*c.* 6 Cl)*	0.01 inactive
1,1,1-trichloro-2-hydroxy-3-(2′-pyridyl) propane*	0.01 inactive
3,5-dichloropicolinic acid*	0.01 inactive
3,4,5-trichloropicolinic acid*	0.01 inactive
methyl tosylate of 2-methylpyridine*	0.01 inactive
methyl tosylate of 2,4-dimethylpyridine*	0.01 inactive
2-[(1′,3′-diketoindan)-2′] pyridine*	0.01 inactive
4-[(1′,3′-diketoindan)-2′] pyridine*	0.01 inactive
2-[(1′,3′-diketoindan)-2′]-4-methylpyridine*	0.01 inactive
2-[(1′,3′-diketoindan)-2′]-6-methylpyridine*	0.01 inactive
nicotine sulfate (from Levine, 1949)	0.00038
sulfates of distilled dipyridyls from pyridine*	0.01
sulfates of distilled dipyridyls from 3° tar bases*	0.01 inactive
sulfates of distilled dipyridyls from denaturing grade bases*	0.01 inactive
acetates of distilled dipyridyls from pyridine*	0.01
crude dipyridyls from 2-methylpyridine*	0.005
distilled dipyridyls from 2-methylpyridine*	0.0025
sulfates of distilled dipyridyls from 2-methylpyridine*	0.005
acetates of distilled dipyridyls from 2-methylpyridine	0.01
1,2-di-(2′-pyridyl)ethane*	0.005
1,3-di-(2′-pyridyl)propane*	0.01 inactive
1,2,3-tri-(4′-pyridyl)propane*	0.04 inactive
sym-di-(2-pyridyl) thiourea*	0.01
2,3,4,5-tetra-(4′-pyridyl)thiophene dihydrochloride*	0.0186 inactive
2,3,4,5-tetra-(1′-octyl-4-′pyridinium)thiophene tetrachloride*	0.01 inactive
2,3,4,5-tetra-(1′-dodecyl-4′-pyridinium)thiophene tetrachloride*	0.0083 inactive
2,3,4,5-tetra-(1′-*n*-hexadecyl-4′-pyridinium)thiophene tetrachloride*	0.007 inactive
2,3,4,5-tetra-(1′-octadecyl-4′-pyridinium)-thiophene tetrachloride*	0.0065 inactive
Reaction product of 2,3,4,5-tetra-(4′-pyridyl)thiophene and 4,6-dinitro-*o*-cresol*	0.0127

Piperidine and its derivatives

Piperidine*	0.01 inactive
2-methylpiperidine*	0.01 inactive
mixed methylpiperidines*	0.01 inactive

*Courtesy of Dr. C. W. Kearns, Department of Entomology, University of Illinois and of Koppers Company, Inc.

**Courtesy of Dr. C. S. Marvel, Department of Chemistry, University of Illinois.

***Courtesy of Reilly Tar and Chemical Corporation.

N-isopropylpiperidine*	0.01	inactive
N-*n*-dodecylpiperidine*	0.0025	
N-nitrosopiperidine*	0.01	inactive
N-nitroso-2-methylpiperidine*	0.01	
1,3-dimethylpiperidine*	0.01	inactive
1,4-dimethylpiperidine*	0.01	inactive
1,2,6-trimethylpiperidine*	0.01	inactive
1-ethyl-2-methylpiperidine*	0.01	inactive
N-*n*-dodecyl-N-isopropylpiperidinium bromide*	0.01	inactive
N-*n*-hexadecyl-N-isopropylpiperidinium bromide*	0.01	inactive
N-*n*-hexadecyl-N-ethyl-2-methylpiperidinium bromide*	0.01	
N-*n*-dodecyl-N-methyl-3-methylpiperidinium bromide*	0.01	inactive
N-*n*-tetradecyl-N-methyl-3-methylpiperidinium bromide*	0.01	inactive
N-*n*-dodecyl-N-methyl-4-methylpiperidinium bromide*	0.01	inactive
Dibenzoyl octahydronicotine**	0.01	inactive
β-naphthyl-β-piperidinoethyl ketone hydrochloride*	0.01	inactive
hexahydronicotine**	0.01	inactive
acetylhexahydronicotine**	0.01	inactive
benzoylhexahydronicotine**	0.01	inactive
phenyl-β-piperidinoethyl ketone hydrochloride*	0.01	inactive
4-methylphenyl-β-piperidinoethyl ketone hydrochloride*	0.01	
2-piperidinomethylcyclohexanone hydrochloride*	0.01	inactive
2-piperidinomethyl-4-methylcyclohexanone hydrochloride*	0.01	
5-oxo-6-piperidinomethyltetralin hydrochloride*	0.01	inactive
1,2-di-(4′-piperidyl)ethane dihydrochloride*	0.01	inactive
1,2-di-(N-ethyl-4′-piperidyl) ethane*	0.01	inactive
Pyrimidine derivative		
1-(*o*-bicyclohexyl)2-thio-4,4,6-trimethyldihydropyrimidine*	0.01	inactive
Piperazine derivative		
1-diethylcarbamyl-4-methylpiperazine dihydrogen citrate (= hetrazan, = caricide)******	0.01	inactive
Pyrrolidine derivative		
N-chlorosuccinimide*****	0.01	inactive
Triazolidine derivatives		
urazole****	0.01	inactive
urazine****	0.01	inactive
Thiazole derivative		
2-amino-5-nitrothiazole (= enheptin T)******	0.01	inactive
Indole derivatives		
N-vinylindole*	0.01	
2,3-dimethylindole*	0.01	
Benzoxazole derivative		
2-aminobenzoxazole*	0.01	inactive
Quinoline derivative		
6-methoxy-8-aminoquinoline**	0.01	inactive
Pyrrocoline derivative		
2-phenylpyrrocoline*	0.01	inactive
Carbazole derivative		
N-vinylcarbazole*	0.01	inactive

****Courtesy of Dr. L. F. Audrieth, Department of Chemistry, University of Illinois.

*****Courtesy of Arapahoe Chemicals, Inc.

******Courtesy of American Cyanamid Corporation.

DISCUSSION

Thirty-six of the 52 pyridine compounds, 24 of the 29 piperidine compounds, and the few pyrimidine, piperazine, pyrrolidine, triazolidine, thiazole, benzoxazole, quinoline, pyrrocoline, and carbazole compounds studied were inactive at the highest concentration tested, which was at least 0.01 M. The two indol derivatives were toxic at a concentration of 0.01 M. It should be worthwhile to test other indol derivatives.

The greatest activity observed was among the pyridine derivatives. The most toxic compound was 2-hexylpyridine, which killed at a concentration of 0.00025 M. Nicotine sulfate was not tested at this concentration but killed at 0.00038 M. Other active compounds in the 2-substituted series were 2-vinylpyridine, 2-vinyl-6-methylpyridine (0.005 M.), and 2-aminopyridine (0.01 M.). The effect of vinyl pyridines on other organisms has apparently not been reported, but studies on the other two compounds have been made. King, Frear, and Dills[12] found 2-*n*-hexylpyridine to be toxic to the bean aphid, *Aphis rumicis*, when used as a contact spray. King and Frear[10] found that when used as a fumigant, it was more active than pyridine against the red spider mite, *Tetranychus telarius*, and less toxic than pyridine against the milkweed bug, *Oncopeltus fasciatus*, and the confused flour beetle, *Tribolium confusum*. It was also somewhat toxic for *T. confusum* when mixed in its diet. 2-aminopyridine was about as toxic for the bean aphid as 2-hexylpyridine when used as a contact spray[12] and more toxic than pyridine against the red spider mite.[9] Baldwin[1] found that

both pyridine and 2-aminopyridine were toxic for *A. lumbricoides*, paralyzing anterior section preparations at a concentration of 1 : 5000. In the present study with the stages of horse strongyles in feces, pyridine was inactive at 0.01 M., 2-aminopyridine was active at that concentration, and 2-hexylpyridine was 40 times as toxic as 2-aminopyridine.

Only two of the ten chlorinated pyridine derivatives were active. These were chlorinated (approximately 4 Cl) 3-methylpyridine (0.0025 M.) and chlorinated (approximately 5 Cl) 4-methylpyridine (0.01 M.).

Because of their relationship to nicotine, the dipyridyls are an interesting group. Most of the dipyridyls and their sulfates and acetates were active. The distilled dipyridyls from 2-methylpyridine were toxic at a concentration of 0.0025 M., which was twice the active concentration of their sulfates or of the crude dipyridyls from 2-methylpyridine. The sulfates of the distilled dipyridyls from 2-methylpyridine, in turn, were twice as active as their acetates or as the sulfates of the distilled dipyridyls from pyridine.

The tests of dipyridyls were carried out on mixtures, since no pure single compounds were available. It is likely that tests of pure dipyridyls would reveal differences in toxicity. The position of the linkage between the two rings has a marked effect on the toxicity of nicotine analogues. Richardson, Craig, and Hansberry[18] found that β-nicotine (which has a 3,2′ linkage). is 15.6 times as toxic for the bean aphid as γ-nicotine which has a 2,2′ linkage). Similarly, Smith, Richardson, and Shepard[22] found with the same aphid that 2,2′- and 2,3′-dipyridyls were twice as active as 3,3′-dipyridyl. The most striking difference was reported by Baldwin,[1] who found that 2,2′-dipyridyl was more than 100 times as toxic for *A. lumbricoides* anterior segments as 2,3′-dipyridyl, while 3,4′- and 4,4′-dipyridyls were inactive at the highest concentration tested.

The insertion of two methylene groups between the pyridine rings to form 1,2-di-(2′-pyridyl)ethane caused little if any decrease in activity, since this compound was toxic at a concentration of 0.005 M. However, insertion of a third methylene group between the rings to form 1,3-di-(2′-pyridyl)propane was followed by a loss of activity. On the other hand, *sym*-di-(2-pyridyl)thiourea, in which there are also three atoms between the pyridine rings, was active at a concentration of 0.01 M.

Only five of the 29 piperidine compounds tested were active. The most toxic was N-*n*-dodecylpiperidine, which killed the strongyles at a concentration of 0.0025 M. The other four compounds were active at a concentration of 0.01 M. Saturation of the pyridine ring of nicotine to form hexahydronicotine was accompanied by loss of activity.

Of interest in this connection are the reports of King and Frear[10] on the milkweed bug and confused flour beetle, and King, Frear and Dills[12] on the bean aphid. They found that 2-*n*-hexylpiperidine was more toxic for the confused flour beetle and the milkweed bug, but less toxic for the bean aphid, than its pyridine counterpart, while 4-*n*-amylpiperidine was more toxic for the milkweed bug but of about the same toxicity for the confused flour beetle and the bean aphid as its pyridine counterpart.

The only thiazole derivative tested was 2-amino-5-nitrothiazole (enheptin T), which was reported by Waletzky, Brandt, Bliznick and Hughes[24] and Waletzky, Clark and Marson[25] to be effective against the protozoon *Histomonas meleagridis* in turkeys. It was inactive against horse strongyle larvae in the highest concentration tested.

The piperazine derivative, caricide, was ineffective against horse strongyle larvae. This is not unexpected. Although this compound is toxic for many filariids, ascarids and Strongyloides, Todd *et al.*[23] found that it was not effective against fourth stage strongyle larvae in the horse and Hewitt *et al.*[7] and Colglazier and Enzie[3] found that it was ineffective against the closely related *Ancylostoma caninum* in the dog.

Parasite Stages Affected.—There are four developmental stages in horse feces—the egg, first stage larva, second stage larva, and third stage larva. The first two larval stages feed on bacteria and other material in the feces while the third stage larva is ensheathed and does not feed.

Eggs were separated from feces by flotation with saturated sodium chloride solution, and larvae of all three stages by the Baermann technique. The first stage larvae were collected after the fecal sample had been allowed to incubate at room temperature for one day, the second stage larvae

after it had incubated two days, and the third stage larvae after it had incubated one week.

All four stages were washed repeatedly with water, and groups of 50 to 100 were treated with 0.01 M. of 2-hexylpyridine in small Syracuse watch glasses (1-in. diameter).

Since this compound is not significantly water-soluble, two series of tests were carried out. In the first series, the compound was dissolved in ether and placed in the watch glass. The ether was allowed to evaporate, leaving a thin layer of 2-hexylpyridine on the bottom of the watch glass. The eggs or larvae were then placed in the watch glass in 1 ml. of water, a coverslip was sealed onto the watch glass with vaseline, and the preparations were observed for five days. Untreated control preparations were also made. Neither the eggs nor the larvae were markedly affected by the compound.

In the second test, the compound was emulsified with 0.5 per cent Triton NE, and the eggs or larvae were placed in 1 ml. of the emulsion as described above. Control preparations in 0.5 per cent Triton NE and in water were also made. In the preparations containing 2-hexylpyridine, the eggs failed to embryonate, and the first and second stage larvae were killed in less than a day. The third stage larvae, however, were not markedly affected even after five days.

SUMMARY

In a study of the effect of 93 N-heterocyclic compounds on the developmental stages of horse strongyles in feces, it was found that 16 of 52 pyridine derivatives, five of 29 piperidine derivatives, and two indol derivatives were toxic at a concentration of 0.01 M. or less. The most active compounds were 2-hexylpyridine, which killed the nematodes at a concentration of 0.00025 M., and nicotine sulfate, which did so at 0.00038 M. Other active compounds included chlorinated (approximately 4 Cl) 3-methylpyridine, mixed dipyridyls from 2-methylpyridine, and N-*n*-dodecylpiperidine (0.0025 M.); 2-vinylpyridine, 2-vinyl-6-methylpyridine, the sulfates of dipyridyls from 2-methylpyridine, and 1,2-di-(2'- pyridyl)ethane (0.005 M.); and 2-aminopyridine, chlorinated (approximately 5 Cl) 4-methylpyridine, the sulfates and acetates of dipyridyls from pyridine, the acetates of dipyridyls from 2-methylpyridine, *sym*-di-(2-pyridyl)thiourea, N-nitroso-2-methylpiperidine, N-*n*-hexadecyl-N-ethyl-2-methylpiperidinum bromide, 4-methylphenyl-*β*-piperidinoethyl ketone hydrochloride, 2-piperidinomethyl-4-methylcyclohexanone hydrochloride, N-vinylindole and 2,3,-dimethylindole (0.01 M.).

Saturation of the pyridine ring of nicotine to form hexahydronicotine was accompanied by loss of anthelmintic activity. Caricide, which is active against filariids and ascarids, was inactive in this study.

Washed eggs and first and second stage larvae were killed by an emulsion of 0.01 M. 2-hexylpyridine in 0.5 per cent Triton NE, but washed third stage larvae were not markedly affected even after five days.

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A Study of Postvaccinal (Hog Cholera) Losses

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In 1916, only eight years after the publication of the classical work of Dorset, McBryde, and Niles,[1] Starr[2] reported unfavorable reactions following hog cholera vaccination. In this report, he suggested that underdosing with hog cholera antiserum was a cause of postvaccination sickness. Later, Conn,[3] Cahill,[4] Heil,[5, 6] and Kernkamp[7] considered bacterial infection (latent, concomitant, or secondary) as a possible cause, while Birch[8] and Kernkamp[7] also recognized the possibility that other conditions may affect the physical well-being of the vaccinated pig. On the basis of experimental results, Cahill[4, 9] maintained that sickness following vaccination was due to some condition other than hog cholera and theorized that an abnormal host-antigen relationship might be responsible. Benner[10] held that impotent hog cholera antiserum, and hog cholera virus were responsible, while Birch[8] urged careful handling of the biological products to prevent loss of potency.

Research conducted by the Bureau of Animal Industry, United States Department of Agriculture (BAI, U.S.D.A.), resulted in the recognition of a variant hog cholera virus[11, 13] as a factor in some of the postvaccinal disturbances of swine in 1949 and 1950. In an effort to prevent such losses, Dale, Schoening, Cole, Henley, and Zinober[14] recommended (a) increased serum dosage in immunization of pigs by the serum-virus method and (b) vaccination before weaning.

During the summer of 1950, postvaccinal reactions accompanied by serious death losses occured in Illinois swine herds. The actual proportion of herds or animals which became affected is unknown. In a limited field survey of herds in which death losses occurred, the mortality varied from 24 to 100 per cent. Necropsy usually revealed evidence of septicemia accompanied by bronchopneumonia or enteritis or both. A majority of the affected animals which were examined postmortem had shown fever and leukopenia. Naturally, the relation of hog cholera to the losses deserved investigation. In an effort to examine this relationship, pig inoculation tests were instituted. The primary purpose of this report is to record the results of inoculating hog cholera-susceptible, hog cholera-vaccinated and serum-protected pigs with blood and other tissues of pigs suffering postvaccinal illness. Secondarily, the results of a cross-immunization test between hog cholera viruses and hog cholera antiserums from two sources are reported.

Materials and Methods

Tissues selected for study were collected postmortem from sick pigs submitted to the diagnostic laboratory of the College of Veterinary Medicine, University of Illinois. Blood, spleen, kidney, and occasionally gall bladder were saved for inoculation purposes. All blood was oxalated except in test V, in which it was defibrinated. All tissues were held at approximately —15 C. until inoculated into experimental pigs three to twenty-seven days later.

Tissue suspensions for animal inoculation were prepared by grinding the tissues in approximately four volumes of physiological saline. Although large tissue particles were removed from the suspension by passing it through several layers of gauze, none of the inoculums was passed through a bacterial filter. Hence, each inoculum (except in tests I and V) was cultured on blood agar and MacConkey's agar immediately prior to pig inoculation to determine the presence or absence of pathogenic bacteria. Contaminating organisms such as *Escherichia coli* and *Micrococcus albus* were frequently isolated, but other organisms generally considered pathogenic for swine were not found.

All inoculations were made subcutaneously into the axillary space. All vaccinated pigs had received hog cholera virus and hog cholera antiserum at least fifty-six days previously. Leukocyte counts were made on oxalated blood obtained from the jugular vein or from the axillary blood vessels when the animals were killed for autopsy.

The inoculated pigs were kept in clean, disinfected pens and precautions were taken to

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avoid introduction of intercurrent infections so far as possible. The pigs were given free access to an adequate ration (University of Illinois 12-W). All inoculated pigs that died or were killed *in extremis* were examined for gross lesions. Liver, spleen, and kidney were routinely cultured on blood agar and MacConkey's agar.

Inoculation Studies

Inoculation Test 1.—Herd 1 suffered postvaccinal illness beginning ten days after simultaneous vaccination with hog cholera virus and hog cholera antiserum. Symptoms included fever, unsteady gait, weakness, and loss of weight. Five milliliters of inoculum containing spleen, gall bladder, and blood, taken at time of autopsy of swine from this herd were injected subcutaneously into each of 3 hog cholera-susceptible pigs. Two of these pigs received simultaneously a minimum protective dose of hog cholera antiserum. Three hog cholera-susceptible pigs from the same lot were vaccinated with regular doses of commercial hog cholera virus and hog cholera antiserum and were confined in a separate pen. One hog cholera-vaccinated pig in a third pen received 5 ml. of the tissue suspension (*see* table 1). Beginning one day before inoculation, white blood cell counts and body temperatures were recorded daily for sixteen days on the 7 pigs.

On the eighth day after inoculation, the susceptible pig that had received only the tissue suspension showed lethargy, diarrhea, fever, and leukopenia. Autopsy following death on the eleventh day revealed lesions considered characteristic of hog cholera. Salmonella was isolated from the liver, spleen, and kidney, and *Pasteurella multocida* was isolated from the lungs.

The 2 susceptible pigs receiving the tissue suspension and hog cholera antiserum simultaneously remained healthy as did the previously vaccinated pig receiving tissue suspension only. Daily leukocyte counts and body temperatures of the 6 remaining pigs did not vary significantly from normal.

Inoculation Test 2.—Oxalated blood was collected from swine of herd 2, which had just become sick although they had been vaccinated with hog cholera virus and hog cholera antiserum four weeks earlier. Five milliliters of the blood were injected subcutaneously into each of 3 susceptible pigs and into 2 pigs which had been previously vaccinated with hog cholera virus and hog cholera antiserum. One of the hog cholera-susceptible pigs received simultaneously 50 per cent more than the minimum recommended dose of hog cholera antiserum (*see* table 1). All 5 pigs were maintained in the same pen throughout the course of the experiment.

The 2 hog cholera-susceptible pigs that received the oxalated blood unaccompanied by hog cholera antiserum showed extreme lethargy, diarrhea, fever, and leukopenia after the sixth day. Both animals became moribund and were killed after the thirteenth day. Autopsy revealed an enteritis but no petechiae of the serous membranes. Salmonella was isolated from the spleen of 1 of the pigs.

The 3 remaining exposed pigs (that is,

TABLE 1—Results of Inoculation Tests

Test no.	Experimental Pigs: Pig no.	Weight (lb.)	Vacc.*	Treatment: HC Antiserum (ml.)	Inoculum (ml.)	Results
	1-12	—	+	—	5 TS	H
	2-1	11	—	20	5 TS	H
I	3-6	14	—	24	5 TS	H
	4-4	12	—	—	5 TS	D
	5-2	16	—	24	2 V	H
	6-3	17	—	24	2 V	H
	7-5	9	—	24	2 V	H
	1-25	47	+	—	5 Bl	H
	2-26	49	+	—	5 Bl	H
II	3-18	76	—	45	5 Bl	H
	4-13	75	—	—	5 Bl	D
	5-21	42	—	—	5 Bl	D
	1-24	48	+	—	5 TS	H
	2-29	42	+	—	5 TS	D
III	3-14	80	—	45	5 TS	H
	4-16	92	—	45	5 TS	H
	5-15	74	—	—	5 TS	D
	6-22	40	—	—	5 TS	D
	1-27	69	+	—	5 TS	H
	2-28	57	+	—	5 TS	H
IV	3-17	66	—	45	5 TS	H
	4-19	59	—	45	5 TS	H
	5-23	39	—	—	5 TS	D
	6-20	51	—	—	5 TS	D
	1-10	40	+	—	3 Bl	H
	2-814	30	+	—	3 Bl	H
	3-813	40	+	—	3 Bl	H
	4-801	39	—	45	3 Bl	H
V	5-802	49	—	45	3 Bl	H
	6-807	63	—	45	3 Bl	H
	7-803	56	—	—	3 Bl	D
	8-805	29	—	—	3 Bl	D
	9-804	43	—	—	3 Bl	S

* = Previous vaccination with hog cholera virus and anti-hog cholera serum. TS = Tissue suspension. Bl = Blood. V = Hog cholera virus. H = Remained healthy. D = Died or was killed when moribund. S = Sickened but survived.

the 2 previously vaccinated pigs and the pig which received hog cholera antiserum simultaneously) did not sicken during the fifteen-day observation period.

Inoculation Test 3.—A saline suspension of spleen, kidney, and gall bladder was prepared from swine of herd 3 which sickened three weeks following vaccination with hog cholera tissue vaccine. Each of 4 hog cholera-susceptible pigs received 5 ml. of the tissue suspension subcutaneously, and 2 of these pigs received 50 per cent more than the minimum recommended dose of hog cholera antiserum simultaneously. Two pigs that had been previously vaccinated with hog cholera virus and hog cholera antiserum received 5 ml. of the tissue suspension (*see* table 1). All 6 exposed pigs were kept in the same pen.

The 2 hog cholera-susceptible pigs which did not receive hog cholera antiserum and one of the previously vaccinated pigs were sick as evidenced by fever and leukopenia after the third day. These 3 pigs became moribund and were killed on the thirteenth to fifteenth days. Lesions considered characteristic of hog cholera were found in all at autopsy. On bacteriological examination, *P. multocida* was recovered from the liver of one of the hog cholera-susceptible pigs.

The other pigs did not sicken visibly throughout the fifteen-day observation period.

The failure of the previously vaccinated pig to withstand the exposure was not considered conclusive evidence that the inoculum contained an infective agent other than hog cholera virus. This pig may have been one of the small percentage of swine that fail to develop immunity following vaccination. The survival of its companion pig supports this viewpoint. Moreover, a part of the tissue suspension prepared for this experiment was included in the composite inoculum of test IV in which 2 previously vaccinated pigs survived.

Inoculation Test 4.—A composite saline tissue suspension of spleen, kidney, and blood was prepared from swine of nine herds. Herds 3 and 4 had been vaccinated with hog cholera tissue vaccine, and herds 2, 7 and 9 had received hog cholera virus and hog cholera antiserum. The type of vaccination used in herds 5, 6, 8 and 10 is unknown. The time interval between vaccination and the onset of visible symptoms was one week in herd 7, nine days in herd 8, ten days in herd 9, three weeks in herds 3, 5, and 6, and four weeks in herd 2. There is no record in this regard for herds 4 and 10.

Each of 4 hog cholera-susceptible pigs and 2 pigs which had been previously vaccinated with hog cholera virus and hog cholera antiserum received 5 ml. of this composite tissue suspension. Two of the 4 hog cholera-susceptible pigs received simultaneously 50 per cent more than the

TABLE 2—Results of Cross-Immunization Test

Experimental Pigs			Treatment				
			ml. Hog Cholera Antiserum		ml. Hog Cholera Virus		
No.	Weight (lbs.)	Previous vaccination	A	B	A	B	Results
1-30	22	—	25	—	3	—	healthy
2-31	20	—	25	—	3	—	healthy
3-40	30	—	30	—	—	3	healthy
4-41	24	—	30	—	—	3	healthy
5-32	20	—	—	25	3	—	healthy
6-33	34	—	—	30	3	—	healthy
7-38	30	—	—	30	—	3	healthy
8-39	17	—	—	25	—	3	healthy
9-34	19	—	—	—	3	—	moribund
10-35	30	—	—	—	3	—	healthy
11-36	26	—	—	—	3	—	died
12-37	22	—	—	—	3	—	died
13-42	22	—	—	—	—	3	died
14-43	28	—	—	—	—	3	moribund
15-44	18	—	—	—	—	3	died
16-45	20	—	—	—	—	3	died
17-811	75*	+	—	—	3	—	healthy
18-812	75*	+	—	—	3	—	healthy
19-810	75*	+	—	—	—	3	healthy
20-3	75*	+	—	—	—	3	healthy

*Estimated.

minimum recommended dose of hog cholera antiserum (*see* table 1). All 6 pigs were maintained in the same pen.

The 2 hog cholera-susceptible pigs which did not receive hog cholera antiserum became clinically sick after the sixth day. One died on the thirteenth day and the other became moribund and was killed on the fifteenth day. Autopsy of these pigs revealed a pronounced bronchopneumonia in 1 of the pigs and congestion of the large intestine mucous membrane in the other. *Pasteurella multocida* was recovered from the liver, kidney, spleen, and lung of the former pig.

The 4 remaining pigs did not appear visibly sick during the fifteen-day period of observation.

Inoculation Test 5.—A composite inoculum was prepared by pooling defibrinated blood collected from swine of six different herds. Herds 11 and 8 had been vaccinated with hog cholera tissue vaccine. There was no record of the type of vaccination in herds 12, 14, 15, and 16. In herd 11 visible illness appeared three months after vaccination. The time interval between vaccination and postvaccinal sickness in herds 12, 13, 14, 15 and 16 is unknown.

Six hog cholera-susceptible pigs and 3 pigs previously vaccinated with hog cholera virus and hog cholera antiserum were maintained in the same pen. Each of these 9 pigs received 3 ml. of the composite blood subcutaneously. Three of the 6 hog cholera-susceptible pigs received simultaneously 50 per cent more than the minimum recommended dose of hog cholera antiserum (*see* table 1).

Two of the 3 hog cholera-susceptible pigs that did not receive hog cholera antiserum became visibly ill five days later. One died on the seventh day. Significant gross lesions included hyperemia of the mucous membrane of the stomach and large intestine as well as diffuse hemorrhages in the mandibular and in most visceral lymph nodes. Petechiae were not seen. On bacteriological examination of liver, spleen, and kidney *Salmonella typhimurium* and *P. multocida* were recovered. The other pig died on the fifteenth day, and lesions of septicemia, including hemorrhages in the skeletal and visceral lymph nodes and petechial to ecchymotic hemorrhages in the cortex and medulla of each kidney were found at postmortem examination. *Salmonella choleraesuis* was recovered from the liver. The third pig manifested slight clinical sickness and eventually recovered. The remaining 6 pigs appeared healthy during the fifteen-day observation period.

Cross-Immunization Test. — Postvaccination reactions were reported to have followed field use of one brand of hog cholera virus and hog cholera antiserum. On the other hand, a combination of hog cholera virus and hog cholera antiserum of another brand had been used in the field without undesirable complications. Corresponding serial numbers of the hog cholera virus and hog cholera antiserum of each brand were used in a cross-immunization experiment. The products are identified as (1) Serum A and Virus A and (2) Serum B and Virus B.

Two groups, each consisting of 8 unvaccinated pigs and 2 hog cholera-vaccinated pigs, were maintained in separate enclosures. Each pig received 3 ml. of one of the viruses. Virus A was used on the pigs of one group and Virus B on the others. Each of 2 of the unvaccinated pigs in each pen received simultaneously 25 or 30 ml. of Serum A, and 2 other comparable pigs received similar doses of Serum B. The smaller dosage was administered to pigs which weighed less than twenty-four pounds. The remaining 6 pigs in each pen received no hog cholera antiserum (*see* table 2).

The pigs were observed for twenty-five days, during which time periodic white blood cell counts and body temperature determinations were made.

None of the unvaccinated pigs that had received either Serum A or Serum B became visibly sick during the course of the experiment. Furthermore, the previously vaccinated pigs remained healthy following exposure to Virus A and Virus B.

Seven of 8 unvaccinated pigs that received either virus A or virus B and no hog cholera antiserum died or became moribund and were killed between the eleventh and nineteenth days. Lesions of septicemia such as petechiae of the kidneys, epiglottis, urinary bladder, and mucosa of stomach and large intestine were found at autopsy. *Pasteurella multocida* was recovered from the liver and lung of 1 pig and from the lung of another. Bacteriological examination failed to reveal the presence of pathogenic bacteria in the remaining 5 pigs. The surviving pig had been exposed to virus A.

The virus control pigs did not die as soon as might be expected for hog cholera-susceptible pigs receiving a parenteral inoculation of fully potent commercial hog cholera virus. This observation suggests the possibility that the unvaccinated pigs used in the experiment carried a passive immunity obtained from the colostrum of their dams. If so, it seems logical to assume that a residual immunity may have enhanced the cross-protection.

DISCUSSION

The results of the inoculation tests described herein strongly suggest that hog cholera virus was responsible for at least some of the losses. Although the presence of a variant strain of hog cholera virus was not demonstrated in the outbreaks studied, neither is that possibility denied. The results of these tests reëmphasize that hog cholera immunity is not absolute; many of the postvaccinal losses are probably due to combinations of factors which (a) prevent development of an adequate immunity following inoculation or (b) cause deterioration of such active immunity later. When hog losses occur, the possibility of hog cholera should not be disregarded simply because animals that become ill have been previously inoculated with appropriate amounts of hog cholera virus and hog cholera antiserum, or with one of the vaccines.

SUMMARY

Typically affected pigs from 16 Illinois herds that suffered heavy postvaccinal losses revealed gross lesions of septicemia at autopsy. Blood and other tissues collected at the autopsy of typically affected pigs from these herds were injected into (1) cholera-susceptible pigs; (2) cholera-susceptible pigs that simultaneously received protective doses of hog cholera antiserum; (3) pigs previously given hog cholera antiserum and hog cholera virus. The results of these inoculations revealed reasonable evidence of the presence of hog cholera virus in each inoculum. This means that (a) where a single herd was represented in an inoculum, hog cholera virus was present in each herd, and (b) where specimens from several herds were combined for inoculation, hog cholera virus was present in one or more of the herds represented in the composite inoculum.

Two brands of commercial hog cholera antiserum and hog cholera virus were used in cross-immunization pig inoculation tests. Field use of one of the brands had been followed by considerable postvaccinal loss. The viruses produced fatal illness in all but 1 nonprotected pig but failed to produce noticeable effect when injected into pigs that had been previously vaccinated. Moreover, all simultaneously treated pigs remained healthy, indicating that each hog cholera antiserum provided protection against each of the viruses.

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Experimental Infection of Sheep and Goats with the Nematode Lungworm, Dictyocaulus Filaria

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Dictyocaulus filaria, one of the pathogenic parasites of sheep and goats, may cause considerable loss of weight and sometimes death of the infected animal. Experimental lungworm infections, using a considerable number of animals under controlled conditions, have been studied previously by two investigators, Kauzal (1933, 1934) and Shaw (1934, 1937). Continued study of the activity of the worms and the responses of the host to infection should lead to a better understanding of lungworm disease and how to control the causative parasites.

Materials and Methods

Twenty-three Shropshire x Hampshire sheep and 2 kids were used in this study. The sheep varied from 2 weeks to thirty-one months of age at the time of infection, and the kids were 2 months old. Unless otherwise noted, the test hosts were free from helminth parasites, except for a few Strongyloides. These animals were fed an adequate diet of alfalfa hay and mixed grain, and a block of salt was always available to them. They were maintained under conditions designed to prevent extraneous infection with helminth parasites. Fifteen lambs were kept in three large pens in a barn with cement floor and sides. Thrice weekly the pens were thoroughly swept and then washed, first with tap water, and then with hot water. The feed was placed in racks raised above the floor, and the slats were spaced so that the sheep could get only their heads through to the feed. The remaining lambs, mature sheep, and the kids were kept in pairs in outdoor pens with cement sides and cement floor raised above the ground. Thrice weekly the feces were h ed out, from outside the pens and then, using a hose, the pens were thoroughly washed with tap water. It was considered that cleaning the pens three times a week was sufficient to prevent larvae that might have been passed in the feces of the animals from reaching the infective stage, which requires at least four days.

The kids were infected with larvae cultured from eggs and from preinfective larvae of *Dictyocaulus filaria* recovered from the lungs of a ram which had died as a result of infection with this parasite. The sheep were fed larvae obtained from the feces of a naturally infected ewe. The larvae were separated from the feces by floating the pellets in water in the Baermann apparatus. They were washed in water several times and allowed to develop to the infective stage on moist, granular, bone charcoal at room temperature, approximately 21 to 27 C. Larvae for experimental infection of the sheep were 13 to 40 days old, average 19½ days old. Larvae more than 20 days old were stored at 4 C., after separation from the cultures. The larvae were suspended in 10 ml. of water and administered to the test hosts by pipetting them down the oesophagus. Only active larvae were used.

Some of the test sheep (tables 1 and 3) were inoculated with one to four doses of larvae in as many days, whereas others (table 2) were given 19 inoculations during a month. The course of each infection was followed by counts of larvae passed in the feces. The clinical symptoms of infection were noted. Fourteen of the animals were necropsied forty-seven to 186 days after infection. The worms were recovered and counted, and the lungs were examined for evidence of pathological conditions.

Infection of Kids.—A 2-month-old kid was fed 4,500 larvae at one time. This animal died of asphyxiation twenty-eight days after infection, death being caused by a mass of worms, with adherent thick mucus, completely blocking the trachea for 2 in., extending anteriorly from its junction with the bronchi. In addition, there were two smaller verminous plugs, one about ½ in. long, blocking the glottis. Approximately 2,800 worms were recovered, of which 30 were in the oesophagus, presumably as a result of having been coughed up. The majority of the worms were mature, although egg-laying had apparently just begun in some of these and others were not yet passing eggs. Eggs were found in the thick mucus around the worms, and in an abundant serous, straw-colored fluid in the lungs. At least one egg contained a motile larva.

Another 2-month-old kid was fed 1,000 larvae at one time. This animal died forty days later. It was cyanotic and pus was present in the

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TABLE 1—Data on Experimental Infection of 14 Lambs with an Average of Approximately 1,660 Dictyocaulus Filaria Larvae, Given in One to Four Doses

Lambs		Larvae Administered		Observations on Larvae Recovered			Symptoms		Observations on Weights		
No.	Age at infection (months)	Total number	Doses*	Larvae first recovered in feces (days after infection)	Peak larval count (per gram of feces)	Period of recovery of larvae (days)	Coughing first noted (days after infection)	Duration of intermittent cough (days)	Weight loss following infection (lb.)	Weeks after infection when maximum weight loss occurred	Weeks required to regain or exceed weight loss
182	0.5	90	1	32	10	20	None	...	Not weighed	...	...
100	2.4	280	3	34	82	70	78	1	Not weighed	...	...
177	4.3	640	2	43	12	53	54	1	Not weighed	...	...
126	4.9	1,100	2	33	71	70	49	5	0.5	5	2
98	5.4	1,200	1	None	...	...	20	36	1.5	4	2
104	3.5	1,215	2	None	...	...	21	112**	0.5	4	2
102	3.6	1,235	3	33	29	57	20	58	4.0	4	2
101	4.3	1,360	2	None	...	...	29	26	7.5	5	7
125	4.3	1,950	1	34	25	81	38	66	3.0	4	2
103	4.7	1,950	4	49	2	1	23	56	5.0	4	11
118	3.6	2,500	1	57	1	40	33	39	3.5	3	4
122	3.6	2,725	2	49	20	30	27	71	3.0	3	3
105	8.0	3,000	1	None	...	...	21	36	3.5	4	5
109	8.0	4,000	1	40	496	28	19	51	10.5	4	11

*Administered at one-dav intervals, except two-day interval for lamb 101. **Still coughing on day of necropsy.

bronchi and bronchioles. Twenty-five *D. filaria*, all non-gravid adults, were recovered.

Short-Time Infection of Lambs in One to Four Days.—Each of 14 lambs, 2 weeks to 8 months old, was fed 90 to 4,000 larvae in one to four doses, in an average of three days. Larvae were first recovered from the feces of 10 lambs (table 1) thirty-two to fifty-seven days after infection. The peak output ranged from 1 to 495 larvae per gram of feces, and occurred thirty-nine to fifty-seven days after infection. Larvae were recovered for a period of one to eighty-one days.

Coughing occurred in 13 of the 14 lambs, beginning as early as nineteen days after infection, and persisting intermittently for one to 112 days. Other symptoms included rapid, shallow breathing, with the head extended and lowered and, occasionally, with the mouth open; rales; and loss of appetite and weight. Eleven of the 14 lambs were weighed at weekly intervals; the remaining 3 were in the pens outside the barn. All 11 lost weight after the infection, the loss ranging from 0.5 to 10.5 lb., centering about the fourth week after infection. The time required to equal or exceed the maximum weight before the loss occurred ranged from two to eleven weeks.

No marked correlation was noted between the number of larvae administered and the degree of infection, as evidenced by the symptoms shown and larval output. However, the highest peak of larval production, 496 per gram of feces, was from the lamb fed 4,000 larvae, which was the greatest number administered. The greatest weight loss, 10.5 lb., also occurred in this animal. Next highest peaks of larval output were from lambs 100 and 126, which had been fed 280 and 1,100 larvae, respectively. Next highest weight losses occurred in lambs 101 and 103, fed 1,360 and 1,950 larvae, respectively. Coughing persisted longest in lamb 104 fed 1,215 larvae, and lambs 122, 125, 102, 103, and 109 in the order mentioned. Larvae were found in the feces for the longest periods in lambs 125, 126, 100, and 102 in the order mentioned.

Eighty-two to 186 days after infection, when all the lambs (table 1), except 104, no longer showed clinical evidence of infection or passed larvae in the feces, 6 lambs were necropsied to determine whether or not there was any residual effect of the infection. Lamb 104 was still coughing up to the time it was killed. Its left lung was cyanotic and showed evidence of pneumonia. Lambs 126, 125, 103, and 109 had one to three functionless, consolidated, purplish areas of the lung, particularly at the tips of the lobes and along the borders, extending for ½ to 2 in. along the border and ¼ to ¾ in. medially. In addition, lambs 103 and 126 had one and two caseous pus accumulations, respectively, approximately 5 mm. in diameter, at the terminal portions of bronchioles. Lamb 103 also had considerable adhesions of the pleura to the body wall and diaphragm. One actice, sexually immature adult *D. filaria* still remained in lamb 125, 186 days after infection. No worms were recovered from the other lambs autopsied. Lamb 105 showed no residual effect of the infection. Since the findings in the necropsied lambs, except lamb 104, were considered to be typical of the condition after recovery, the remaining lambs were not killed.

Long-time Infection of Lambs During a Month.—Three lambs (table 2), 8.3 to 8.6 months at the time of first infection, were fed a total of 950 larvae each, in 19 doses of 50 larvae, during a period of thirty days. Lamb 111 showed no symptoms, and the other 2 were noticed coughing on only one day. Lamb 132 did not gain any weight from the second to the fifth week after infection, but the other 2 continued to gain weight. Larvae were recovered from the feces of only lamb 129 for fifteen days, beginning forty-three days after the first inoculation, the number never exceeding 1 per gram of feces. Lamb 129 was necropsied forty-seven days after the final inoculation, and five sexually immature adult *D. filaria* were recovered; 1 worm was embedded in dense mucus containing a few blood cells. There was a functionless, consolidated, purplish area of approximately 1 sq. in. in the middle lobe of the left lung. Since neither lamb 111 nor lamb 132 had passed larvae in the feces, and lamb 132 was noticed coughing on only one day, whereas lamb 111 had never shown any symptoms, these 2 animals were not necropsied.

Infection of Adult Sheep.—Five adult sheep (table 3), 4 of which had been used the previous year in an experiment with the intestinal

TABLE 2—Data on Experimental Infection of 3 Lambs with 950 Dictyocaulus Filaria Larvae in 19 Doses of 50 Larvae each, Administered in Thirty Days

Lambs		Observations on Larvae Recovered			Symptoms	
Number	Age at infection (months)	Larvae first recovered in feces (days after infection)	Peak larval count (per gram of feces)	Period of recovery of larvae (days)	Coughing first noted (days after infection)	Duration of cough (days)
111	8.6	None	...	...	None	...
129	8.4	43	1	15	43	1
132	8.3	None	...	...	40	1

parasite, *Oesophagostomum venulosum*, were 17 to 31 months old at the time of infection with *D. filaria*. They were fed 275 to 3,800 larvae in one to three doses in an average of two days. Three of the sheep, 911, 968, and 57, began to cough forty-one to fifty-one days after infection; sheep 911 and 968 coughed only one day, and sheep 57 coughed intermittently over a period of fifty-one days. The other 2 sheep did not show symptoms. Larvae were recovered from 3 sheep, 911, 57 and 62, the average peak being 7 larvae per gram of feces. The patent period ranged from fifteen to twenty-three days.

These sheep were necropsied seventy-three to 259 days after infection. Four to 33 *Oe. venulosum* were still present in the cecum of each of the sheep previously infected with this parasite. Sheep 911 and 62 had two functionless, consolidated, purplish areas near the tips of lobes of the lungs. Contiguous to one of these areas, in sheep 911, there was a caseous pus accumulation containing the remains of an adult *D. filaria*. This sheep had three additional small pus pockets, one containing the remains of an adult *D. filaria*. Sheep 968 also had a caseous pus accumulation, 4 mm. in diameter. Active, sexually immature adults of *D. filaria*, one-fifth to one-fourth the usual size of an adult, were recovered from sheep 62, 911, 968, and 941, the numbers being 3, 4, 10, and 28, respectively. No worms or residual evidence of infection were found in sheep 57.

Sheep 67, which had been fed 100 *D. filaria* larvae when 6 months old, and 500 *Oe. venulosum* larvae when 11 months old, but developed no symptoms of lungworm infection, was fed 4,100 *D. filaria* larvae in two doses on consecutive days when 19 months old. No larvae were recovered from the feces of this animal, nor was it noted coughing. It died of enterotoxaemia sixty-five days after infection. Four sexually immature adult *D. filaria* and 111 mature *Oe. venulosum* were recovered.

Under natural conditions, there are circumstances when the resistance of adult and old sheep breaks down, or perhaps some sheep never build up resistance. The 10-year-old, naturally infected ewe which served as a source of larvae for this study showed clinical evidence of infection and passed larvae for at least five and one-half months. Although this ewe was maintained under conditions designed to prevent extraneous infection with helminths during this period, the larval output rose in the last two months prior to death, and reached a peak ten days before death. At postmortem examination, the lungs were found to contain many large caseous pus pockets. There was excessive mucus and fluid in the trachea and pus in the bronchi. There were extensive adhesions of the pleura and lungs, and fluid in the pleural cavity. Although an adequate diet had been available, the animal was emaciated. Fifty-one mature *D. filaria* were recovered from the trachea, bronchi, and bronchioles. There also were a few Muellerius in the lungs. These had also infested the animal for at least five and one-half months.

DISCUSSION

The clinical and postmortem findings and the time of the occurrence of symptoms in 23 sheep and 2 goats experimentally infected with *D. filaria* were similar to those noted by Kauzal (1933, 1934) and by Shaw (1934, 1937).

One of the primary purposes of the work here reported was to determine the effect on lambs of a moderate number of larvae, administered in a few days, and to ascertain the extent to which growth of the lambs would be delayed as a consequence thereof. Lambs were weighed at weekly intervals or an average of twenty-one weeks before they were infected, and for an average of seventeen weeks after regaining the loss in weight as a result of the infection. Thus, data were obtained on weight loss after infection and the time required to re-

TABLE 3—Data on Experimental Infection of 5 Mature Sheep with an Average of Approximately 2,000 Dictyocaulus Filaria Larvae Given in One to Three Doses

Sheep		Larvae Administered		Observations on Larvae Recovered			Symptoms	
Number	Age of infection (months)	Total number	Doses*	Larvae first recovered in feces (days after infection)	Peak larval count (per gram of feces)	Period of recovery of larvae (days)	Coughing first noted (days after infection)	Duration of intermittent cough (days)
911	31	275	1	35	1.5	20	51	1
968	30	1,035	2	None	...	...	50	1
941	31	2,000	1	None	...	...	None	...
57	17	3,300	3	38	0.5	15	41	51
62	19	3,800	1	43	19	23	None	...

*Sheep 968 dosed on successive days and sheep 57 in four days.

gain the weight so lost. In the preinfection period almost all the lambs gained weight regularly; on rare occasions a lamb lost a pound or two, which loss was regained in a week or two, and the animal continued normal growth thereafter. After infection, however, the lambs lost an average of 3.9 lb., and from two to eleven weeks were required to regain this weight. The loss in weight usually occurred in the fourth week after infection when many of the worms were just reaching maturity and had become large enough, therefore, to do considerable damage. After the lambs succeeded in coughing up the worms and overcoming the irritation that these parasites produced, weight gains again returned to normal.

When larvae were administered in small doses over a relatively long period, the lambs did not appear to become as heavily infected as when comparable numbers of larvae were administered in a comparatively short time. Lambs infected over the longer period showed clinical symptoms for a shorter time and passed fewer larvae than those given comparable numbers of infective larvae in the course of a few days. Moreover, the lambs showing lighter symptoms lost no weight.

Mature sheep, maintained since birth under conditions designed to prevent extraneous acquisition of helminths, were more resistant to an initial infection with lungworms than lambs similarly protected. Fewer mature sheep showed clinical symptoms, they were sick for shorter periods, and they passed fewer larvae than lambs more or less similarly exposed to lungworms.

Some lambs and adult sheep apparently resisted the initial infection better than others. Some of the resistant animals gave evidence of infection by coughing, but lungworm larvae were not recovered from their feces. Another observation that is considered evidence of resistance was the recovery of small adult but sexually immature worms long after they normally would reach maturity. Larvae were not recovered from the feces of 3 mature sheep that harbored such immature adults. Lastly, the lack of marked correlation between the number of larvae administered and the degree of the resultant infection is evidence that there was marked individual variation in resistance of sheep to lungworm infection.

SUMMARY AND CONCLUSIONS

Two kids, 2 months old at the time of infection with a single dose of 1,000 and 4,500 larvae, respectively, died as a result of this parasitism.

Fourteen lambs were infected with an average total of 1,660 larvae in one to four doses in as many days. Larvae were first recovered from the feces of 10 lambs thirty-two to fifty-seven days after infection. The peak larval output for this group averaged 75 larvae per gram of feces and occurred thirty-nine to fifty-seven days after infection. The patent period lasted, on the average, forty-five days.

The lambs showed the following evidence of infection: (1) intermittent coughing, beginning as early as nineteen days after infection and continuing for an average of forty-three days; (2) rapid, shallow breathing, occasionally with the mouth open; (3) rales; and (4) loss of appetite and weight. Eleven lambs, weighed at weekly intervals, lost from 0.5 to 10.5 lb. as a result of the infection. The loss usually occurred in the fourth week after infection, and two to eleven weeks were required to regain or exceed the maximum weight before the loss occurred.

Postmortem findings included: (1) lungworms embedded in dense mucus containing a few blood cells and surrounded by serous, straw-colored fluid; (2) a cyanotic and pneumonic condition; (3) consolidation and atrophy of portions of the lungs, particularly along the borders and at the tips of the lobes; (4) small accumulations of caseous pus, occasionally containing the remains of an adult *Dictyocaulus filaria* at the terminal portions of the bronchioles; and (5) adhesions of the pleura to the body wall and diaphragm.

Three lambs infected by multiple inoculation with 950 larvae administered in 19 doses a month showed little evidence of infection.

Adult sheep, maintained since birth under conditions designed to prevent extraneous infection with helminths, and inoculated with an average of approximately 2,000 larvae in one to three doses over a period of one to four days, were not nearly as susceptible to infection as the similarly infected lambs.

Several lambs and adult sheep were able to resist an initial infection better than the others. In 4 animals, active, sexually immature adults, one-fifth to one-fourth the usual size, were recovered as long as eighty-three to 186 days after infection, long after the worms usually reach maturity. Larvae were never recovered from the feces of 3 mature animals of the above group having these immature worms. Four of the 14 lambs infected over a period of one to four days showed evidence of infection by coughing, but larvae were never recovered from their feces. The same was true of 1 of the mature sheep similarly infected and 1 of the 3 lambs infected with 19 doses over a period of a month. Besides the above evidence, the lack of marked correlation between the number of larvae administered and the larval output and duration of symptoms may be considered evidence of variation in individual resistance.

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A Semen Study of Goats

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SINCE THE PRACTICAL application of artificial insemination of animals, especially dairy cattle, there has been a growing interest and necessity for more knowledge concerning the semen characteristics of farm animals. This is especially true of those for which little is known or in which artificial insemination has not been highly successful. Little information seems to be available on the semen characteristics of goats and, as far as is known, artificial insemination has been employed only experimentally with these animals in the United States. However, artificial insemination of goats in Europe seems to have reached a stage of economic importance.

Götze[1] states that in the province of Hannover, Germany, during 1942-45, around 3,000 goats were inseminated with a fertility of 95 per cent. This rate was not higher than by natural mating.

Möckel[2] reported that the volume of semen from 70 ejaculates of 6 bucks varied from 0.1 to 1.25 cc. with an average of 0.58 cc. Rollinson[7] presented data on a British Saanen which gave an ejaculate of 0.5 cc., with a concentration of 4.2 billion spermatozoa per cubic centimeter and 14 per cent of abnormal spermatozoa. Phillips et al.[5] gave the results on 2 bucks which were observed throughout one year. There was a tendency toward a seasonal effect on the various semen characters observed, especially volume and concentration. This finding was in agreement with the results from 12 rams observed during the same period. Other investigators estimated the average volume of buck semen to be from 0.9 to 1.5 cc. per ejaculate. Perry[4] gave the average concentration of goat semen as 2 billion or more spermatozoa per cubic centimeter. Apparently nothing is available on the morphology of buck spermatozoa.

Because of the little information concerning goat semen, the following observations were made to obtain data on the various semen characteristics of several bucks observed over a considerable period of time. By the information thus obtained, a better evaluation of the practicability of artificial insemination for goats may be gained.

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DESCRIPTION OF EXPERIMENT AND METHODS USED

Semen collections from 9 Toggenburg and 5 Common American bucks were begun in August, 1948. In October of the same year, another Toggenburg was added. Common American goats are of mixed origin and have been bred for many years without the introduction of outside blood. They are smaller in size than the Toggenburgs. Young bucks were added from time to time as they became sexually mature and some of the older ones died. Only bucks which had eight or more collections during a twelve-month period have been included in the analysis of the data. Semen collection was made at four-week intervals. Thus, there were 13 collection periods during the twelve months from August to August. Six of the bucks completed three years, 6 others completed two years, and 7 others one year. In the analysis of variance for two years, the 6 bucks completing three years were also included.

Collection was made by the use of an artificial vagina, such as is used for the ram, and also by a spayed doe brought into estrus by subcutaneous injections of estradiol benzoate or stilbestrol. A few does which had come into estrus naturally were also used. Semen volume was measured immediately by means of a graduated vial. Motility was determined by placing a drop of semen on a glass slide and examining it under the microscope. Motility was scored 1 to 6, 1 being the highest degree and 6 being zero. A small drop of normal saline solution was then put on the slide with the semen and a smear made by drawing a glass slide gently over the solution, spreading it out evenly and thinly for counting abnormal spermatozoa at a later time.

After the samples were brought into the laboratory, semen concentration was determined in the regular manner with the blood-counting chamber, by means of which the number of spermatozoa per cubic centimeter was obtained. The smears made at the barn for determining percentage of abnormal spermatozoa were cleared in chlorazene solution and stained with Ziehl-Neelsen's carbol fuchsin. The number of abnormal spermatozoa out of 500 counted was determined. An analysis of variance was made for each character studied. Variance was calculated separately for the groups which were under observation for one, two, and for three years. The group under observation for two years included those which

were also observed the third year. In the one-year group, there is no year-to-year variance and no interaction where years are involved. When data were missing for a collection period, values were supplied by the method proposed by Allen and Wishart and modified by Yates as given in Snedecor.[6] The correlations (r) between the different characters were calculated from actual data, which included 434 observations for most of the characters correlated.

RESULTS AND DISCUSSION

The averages for each character observed for all bucks combined is shown by years, periods of the years, and season in tables 1 and 2. Period 1 in every case started with August and successive periods followed at four-week intervals. Table 3 shows the characteristics for individual bucks. In the analysis of variance in Table 4, it will be noted that variability in all the semen characters observed was significant as a result of variability between bucks.

Semen Volume.—The volume of semen produced by the bucks in this study (0.65 cc.) is somewhat higher than that reported by Möckel[2] and Rollinson.[7] In a few in-

TABLE 1—Year and Period Averages for Semen Characteristics Observed in Goats in Indicated Number of Observations(n)

Period	n	Volume (cc.)	Motility score	Concentration billions per cc.	Total spermatozoa billions	Abnormal spermatozoa (%)
			1948—1949			
1	11	0.919	2.00	2.318	2.038	5.67
2	11	0.864	1.73	1.285	1.104	9.00
3	12	0.808	1.50	2.082	1.681	3.63
4	12	0.817	1.42	1.834	1.486	4.37
5	12	0.850	1.08	2.374	1.897	4.57
6	12	0.850	1.33	2.052	1.546	5.87
7	11	0.691	1.66	2.765	1.913	8.00
8	12	0.808	1.08	2.552	2.045	11.47
9	12	0.575	1.25	2.536	1.451	9.03
10	10	0.330	1.33[*1]	2.900[*1]	0.863[*1]	8.82
11	11	0.427	1.36	3.039	1.218	9.45
12	11	0.727	1.00	3.646	2.481	9.67
13	12	0.633	2.00	2.443	1.910	30.05
Average	149	0.720	1.44[*2]	2.501[*2]	1.699[*2]	9.23
			1949—1950			
1	9	0.578	1.00	1.704	1.111	9.71
2	12	0.708	1.17	2.055	1.477	6.10
3	11	0.900	1.73	2.376	2.091	6.24
4	11	0.591	1.36	1.962	1.118	8.09
5	11	0.700	1.91	1.748	1.230	10.25
6	12	0.833	1.50	2.645	1.827	7.85
7	12	0.600	2.36[*3]	2.580[*3]	1.642[*3]	6.55[*3]
8	11	0.482	3.18	3.565	1.794	10.15
9	8	0.512	2.50	3.521	1.976	9.10
10	11	0.391	2.73	3.369	1.213	16.47
11	10	0.280	3.00	2.880	0.679	12.40
12	10	0.740	1.60	3.253	2.425	11.16
13	10	0.710	1.70	3.848	2.511	10.14
Average	138	0.623	1.97[*4]	2.661[*4]	1.617[*4]	9.49[*4]
			1950—1951			
1	12	0.592	1.00	2.725	1.698	3.37
2	13	0.800	1.00	2.474	1.941	2.48
3	13	0.785	1.46	2.426	2.055	8.18
4	13	0.608	1.00	2.554	1.544	6.97
5	13	0.615	1.38	2.802	1.675	5.83
6	12	0.733	1.33	2.804	1.830	8.55
7	10	0.640	1.50	2.904	1.712	10.14
8	13	0.415	1.08	3.974	1.222	6.91
9	9	0.489	1.22	3.632	1.613	5.91
10	7	0.329	1.00	3.701	1.147	3.63
11	11	0.545	1.00	3.772	1.715	7.75
12	12	0.433	1.00	3.357	1.354	6.35
13	11	0.718	1.00	2.441	1.776	11.29
Average	149	0.604	1.15	3.004	1.653	6.73
Total Average	436	0.650	1.51[*5]	2.724[*6]	1.658[*6]	8.46[*6]

[*1]n = 9; [*2]Total n for year = 148; [*3]n = 11; [*4]Total n for year = 137; years = 434; [*6]Total n for 3 years = 435.

stances, certain bucks in this study ejaculated less than 0.1 cc. and rarely did the ejaculate exceed 1.0 cc. One buck (76), a half Toggenburg, averaged slightly more than 1.0 cc. His twin brother (77) averaged only 0.5 cc. semen. Two other twin brothers (159 and 160) also showed individual differences in semen volume, averaging 0.3 and 0.5 cc., respectively. One buck (40) was in the experiment for the three years of this study but during the first two years gave fewer than eight ejaculates during the year and, therefore, his data were not included with those for the first two years. However, during the third year, he gave nine ejaculates and has been included with those having one-year records. This buck was eager to serve and would mount the doe and thrust but failed to ejaculate. When he did succeed, the volume averaged only slightly over 0.1 cc. Variance of semen volume was highly significant between bucks and between periods, regardless of whether the bucks were observed for one, two, or three years. Year-

TABLE 2—Averages for Semen Characteristics by Seasons of the Year (3 Years Combined)

Season*	No. of observations	Volume (cc.)	Motility score	Concentration billions per cc.	Total spermatozoa billions	Abnormal spermatozoa (%)
Fall	108	0.740	1.42	2.256	1.650	6.43
Winter	105	0.672	1.64*[1]	2.830*[1]	1.729*[1]	8.34*[1]
Spring	122	0.487	1.57*[2]	3.281*[2]	1.518*[2]	9.32
Summer	101	0.727	1.40	2.449	1.759	9.71
Combined Average	436	0.650	1.51	2.724	1.658	8.46

*Season divisions approximately follow the calendar. The first spring collection falls approximately on the beginning of spring, making four collection periods in this season instead of three as in the others. Fall corresponds to periods 3, 4, and 5 of table 1.
*[1]n = 104; *[2]n = 121.

TABLE 3—Averages of Semen Characteristics of Individual Bucks in Indicated Number of Observations

Buck No.	n	Volume (cc.)	Motility score	Concentration billions per cc.	Total spermatozoa billions	Abnormal spermatozoa (%)
3-year group						
3 B	37	0.854	1.65	2.322	1.791	11.09
9 B	36	0.750	1.03	3.165	2.413	6.15
23 B	34	0.770	1.24	2.534	2.026	7.26
41 B	37	0.613	1.64*[1]	2.642*[1]	1.551*[1]	7.85
43	39	0.608	1.69	2.228	1.311	7.34
47	38	0.453	1.53	2.672	1.094	7.99
3-year Average	221	0.671	1.47*[3]	2.601*[3]	1.694*[3]	7.96
2-year group						
10 B	23	0.574	1.22	3.212	1.889	7.37
17 B	19	0.610	1.06*[2]	2.790*[2]	1.656*[2]	9.03*[2]
26 B	26	0.796	2.77	0.851	0.623	12.85
28 B	26	0.888	1.62	1.945	1.768	9.29
35 B	22	0.577	2.00	3.056	1.633	15.02
52	20	0.625	1.45	3.999	2.500	9.58
2-year Average	136	0.690	1.73*[4]	2.548*[4]	1.640*[4]	10.59*[4]
1-year group						
40	9	0.133	2.00	2.978	0.373	6.84
56	9	0.678	1.00	3.507	2.183	3.53
76	13	1.054	1.00	2.632	2.772	5.74
77	13	0.500	1.00	3.975	1.890	4.37
157	10	0.350	1.20	3.529	1.211	6.44
159	13	0.315	1.31	3.506	1.048	7.78
160	12	0.500	1.33	3.421	1.337	8.32
1-year Average	79	0.520	1.24	3.369	1.587	6.20

*[1]n = 36; *[2]n = 18; *[3]n for total = 220; *[4]n for total = 135.

to-year variance was also significant for those completing two and three years.

Motility.—Motility of spermatozoa seems to be largely an individual characteristic, and can be affected by the health or condition of the individual at time of collection. The technique in handling the semen also may affect motility. In this study, the individuals which showed a low motility (the higher numerical value) were usually defective in some respect. Buck 26B consistently gave semen that was of lower motility and much thinner than that of the other bucks. His semen was frequently stringy in consistency and contained a large percentage of epithelial cells. On slaughter at the close of two years of observation, he was found to have adhesions of the right testis to the scrotal skin. The testicle was very coarse in structure and appeared to be a mass of fibrous connective tissue. The epididymis and vas deferens were abnormal in the same respect. This condition may have been due to an injury early in life. The left testis, epididymis, and vas deferens appeared normal and contained normal spermatozoa. Buck 35B was in poor physical condition for a time, which apparently affected the quality of his semen. The variances for motility are not consistent and probably are of little significance. Where significance is indicated, it is possible that the two bucks, 26B and 35B, having a low motility score for several periods in 1949 and 1950, may have biased the results for the two-year group.

Concentration.—Number of spermatozoa per unit volume of semen appears to be an individual characteristic and influenced by time of year in which collection was made. The figures given by Perry[4] of 2 billion spermatozoa per cubic centimeter and by Rollinson[7] of 4.2 billion are within the range of the findings of the present study. Buck 26B mentioned above as having a defective testicle had a low concentration of only 0.8 billion per cubic centimeter, which probably was due to his defect. Another buck (28B) with a consistently low concentration had but one testicle. He was normal in other semen characteristics and it seems probable that his low concentration of spermatozoa was a result of this anatomical defect.

One observation was a negative relationship between semen volume and concentration as shown by the correlation of —.231 (table 6). Concentration of spermatozoa showed a consistently significant difference between bucks and periods, but not between years, in the analysis of variance. Buck by year interaction, however, was significant.

Total Spermatozoa.—Total number of spermatozoa is the product of volume and concentration. The correlation between volume and total number of spermatozoa was 0.680, while the correlation between concentration and total spermatozoa was 0.413. Apparently bucks with an ejaculate containing more than 1 billion spermatozoa are fertile. Buck 26B with an average of only 0.6 billion total spermatozoa sired triplets, but they were small and weak and died shortly after birth. This, however, may have no relation to the influence of the buck. Buck 40, with an average of approximately 0.4 billion total spermatozoa and because of his poor record in other respects, was considered sterile and was not used for breeding. Buck 56 was shy and gave infrequent service when semen samples were collected,

TABLE 4—Summary of Analysis of Variance of Semen Characteristics in Bucks Which Had One, Two and Three Years of Observations

	Volume			Motility			Concentration			Total spermatozoa			Abnormal spermatozoa		
	3 yrs.	2 yrs.	1 yr.	3 yrs.	2 yrs.	1 yr.	3 yrs.	2 yrs.	1 yr.	3 yrs.	2 yrs.	1 yr.	3 yrs.	2 yrs.	1 yr.
Bucks	**[1]	**	**		**	*	**	**		**	**	**	*	*	*
Periods	**	**	**				**	**	*		*				*
Years	**	*	—[2]	*		—			—			—			—
Interactions															
Bucks X periods															
Bucks X Years*			—			—	**	*	—	*	*	—			—
Periods X years			—			—			—			—	**	*	—
Bucks X period X year			—			—			—			—			—

1**variance highly significant 1%; *variance significant 5%; 2—indicates no interaction for the one-year group.

but his semen was of good quality and he was the sire of several kids.

Percentage of Abnormal Spermatozoa.—The percentage of abnormal spermatozoa varied greatly between bucks. In some cases (9B, 17B, 35B) it was obvious that the condition of the bucks at time of collection had an influence on the percentage of abnormal spermatozoa. The types of abnormalities also were variable but were present in the semen of all the bucks studied. In table 5, the percentages of the different types of abnormal spermatozoa are given for individual bucks. Head abnormalities included macro- and micro-spermia, pear-shaped heads, heads with protoplasmic extrusions, etc. Neck abnormalities included separation of the heads from the spermatozoon body, and attachment of the neck to one side of the head rather than at the center. Middle-piece abnormalities consisted of protoplasmic beads and sharply bent or broken bodies. Tail abnormalities consisted of sharply bent or broken tails and coiled tails. Few types of abnormality other than the ones named were observed. Head abnormalities were lowest in all the bucks. Next came tail abnormalities which, on the average, were more than five times as great as head abnormalities. Middle-piece abnormalities exceeded tail abnormalities by about 1.5 times, and neck abnormalities were the most numerous, averaging nearly one-half the total abnormalities.

TABLE 5—Types of Abnormal Spermatozoa in Bucks (Per Cent)

No. Buck	No. obser.	Head	Neck	Middle pieces	Tail	Total
3 B	37	0.216	5.778	3.097	1.994	11.086
9 B	36	0.317	3.144	1.672	1.006	6.139
23 B	34	0.188	2.753	2.653	1.665	7.259
41 B	37	0.243	3.265	1.903	2.443	7.854
43	39	0.220	3.149	2.554	1.415	7.338
47	38	0.168	3.195	2.579	2.047	7.989
10 B	23	0.243	3.722	2.383	1.026	7.374
17 B	18	0.256	3.656	3.700	1.422	9.033
26 B	26	0.808	7.699	2.900	1.477	12.854
28 B	26	0.685	5.108	2.100	1.400	9.292
35 B	22	1.018	8.691	3.764	1.545	15.018
52	20	0.230	4.690	3.410	1.250	9.580
76	13	0.077	3.585	0.769	1.308	5.738
77	13	0.077	2.031	0.862	1.400	4.369
56	9	0.200	1.089	0.600	1.644	3.533
40	9	0.356	3.489	1.467	1.533	6.844
157	10	0.040	2.960	1.140	2.300	6.440
159	13	0.092	3.831	1.754	2.108	7.785
160	12	0.067	3.633	1.100	3.517	8.317
Ave.	435	0.310	4.118	2.351	1.677	8.457

Randall and Friedlaender[6] pointed out that the neck is the most fragile portion of the spermatozoon and may be broken in handling the semen for smears. Salisbury *et al.*[8] also mentioned that the method of making smears affects the number of morphologically abnormal spermatozoa. The method used in making the smears in this study was the one usually employed and considered most satisfactory. Moench and Holt[3] considered head abnormalities to be the most closely concerned with sterility and low fertility in human beings. They considered semen with as high as 19 per cent head abnormalities to be normal and of good fertilizing quality. If this holds true for buck semen, all the bucks were well within the range for normal fertility.

The consistent differences in percentage of abnormal spermatozoa and in total spermatozoa were due almost wholly to the individual differences between bucks as is shown in the analysis of variance table.

Correlations were calculated between some of the characters which seemingly would be rather closely related. Percentage of abnormal spermatozoa apparently affects motility as shown by the negative correlation of —.378. All the correlations except the one between concentration and percentage of abnormal spermatozoa were highly significant. Partial correlations also were calculated for total spermatazoa and for abnormal spermatozoa, each correlated with concentration, when volume was held constant. The same two characters were also correlated with volume when concentration was held constant. Of the partial correlations, all but the one between abnormal spermatozoa and concentration with volume constant were highly significant. These correlations are shown in table 6.

TABLE 6—Correlations Between Various Semen Characteristics in Bucks

Characters Correlated	Correlation Value
Volume and concentration	—.231**[1]
Volume and total sperm	.680**
Concentration and total sperm	.413**
Concentration and percentage abnormal sperm	—.061
Motility and percentage abnormal sperm	—.378**
Volume and percentage abnormal sperm	—.136**
Volume and (concentration and total sperm)	.799**
Volume and (concentration and abnormal sperm)	—.096
Concentration and (volume and total sperm)	.875**
Concentration and (volume and abnormal sperm)	—.154**

[1] **values highly significant.

Effects of Year, Period, and Season.—It will be noted in table 1 that the averages for the different semen characteristics vary from year to year. This is significant, however, only for semen volume as shown by the analysis of variance in table 4. Periods of the year during which the collections were made have a more marked effect, influencing all the semen characters observed except motility. The influence was greatest on semen volume and concentration in which the analysis of variance due to period was highly significant. If periods 1 to 7, inclusive (corresponding to August through January), are combined, and periods 8 to 13 (corresponding to February through July), differences in these divisions of the year are especially noticeable. These divisions correspond approximately to the normal breeding and nonbreeding seasons, respectively. Averages for the two divisions respectively were: for semen volume, 0.74 and 0.54 cc.; motility, 1.44 and 1.60; concentration of spermatozoa, 2.299 and 3.270 billion per cubic centimeter; total spermatozoa, 1.672 and 1.639 billion; and percentage of abnormal spermatozoa, 6.63 and 10.80. On a seasonal basis of approximately three months corresponding to fall, winter, spring, and summer, there is a close agreement with the data of Phillips *et al.*[5] who made similar studies on rams. Comparing the two species, semen volume was greatest in fall and summer, and lowest in spring, for both bucks and rams. For both species, motility score was highest in the fall and summer and lowest in winter. For bucks, concentration was greatest in the spring and winter while, in rams, it was greatest in the spring and summer. Both were in agreement on being lowest in the fall. The negative correlation between volume and concentration found in buck semen also was evident for the ram semen. Total spermatozoa was greatest in summer and fall for the rams and in summer and winter for the bucks. Percentage of abnormal spermatozoa was greatest in summer and spring for both species, and lowest in the fall.

A possible reason for the few discrepancies between the seasonal effect on buck and ram semen may be due to the fact that the bucks were being used in an experiment to induce spring breeding by means of light control. The bucks were under prolonged hours of lighting by the use of artificial lights from about December 1 to March 1, when the hours of natural lighting were reduced below normal for that period of the year. The bucks were changed monthly, so that most of them came under these modified seasonal conditions. Most of the does under this treatment bred in May and June. Under normal conditions, however, the fall and winter seasons appear to be somewhat more favorable to fertilization and reproduction than the spring and summer months.

Another suggestion of seasonal influence was in the percentage of abnormal spermatozoa. Exceedingly high percentages occurred five times in July, twice in February and May, and once each in April and October. The general activity of the bucks also was noted to be less through the spring and summer months. Every buck on observation gave service in September. In October, December, and February, there was one failure each to give service. In November, June, and July, 2 bucks failed; in August, January, and May, there were 4 failures each, and 8 and 9 in March and April, respectively. In some cases, the inactivity may not have been entirely the fault of the bucks, as the does used for teasing were not always in a proper degree of estrus to excite the bucks.

Effect of Age of Buck.—All the bucks with the letter B following the number were born in February, 1947. These were purebred or very high-grade Toggenburgs. Bucks 40 through 56 (Common American) were born in May and June, 1947. Bucks 76 and 77 (half Toggenburg) were born in November 1948 and the remaining three (157, 159, and 160) were born in December, 1949. Both the two-year and three-year groups were under observation during 1948-1949 and 1949-1950. The three-year group in addition was under observation in 1950-1951, the same year as those having only one year of observation. All the bucks except the 3 born in December, 1949, were more than a year old when semen collections were begun. The 3 mentioned were 8 months old. An analysis of variance of the different semen characteristics due to age of bucks gave significant results only for motility, significant at the 1 per cent level, and percentage of abnormal spermatozoa, significant at the 5 per cent level.

Effect of Weight and Breeding of Buck.—These two factors are rather closely related, since in the recognized breeds there has

been more or less selective breeding with a resulting uniformity of size and conformation. In the Common American goat, which is more mixed in its breeding, there has been less selection and a consequent wider range for individual buck weight. In the group of bucks used there was a difference in weight of 14 lb. between Toggenburg and Common American bucks of similar age. An exception to this in the yearling group is apparently due to the 3 young bucks mentional previously. With the same exception, there was no significant variation in buck weight within the breed.

Differences in semen characteristics due to breeding, as shown by an analysis of variance, are highly significant for semen volume, concentration, and total spermatozoa, and significant at the 5 per cent level for percentage of abnormal spermatozoa. Whether these differences are really a breed characteristic or whether they are due to a difference in the weight of the bucks is rather difficult to determine. It seems, however, that weight may be a largely responsible factor. In the Toggenburgs, the low correlation (0.018) between semen volume and buck weight possibly may be explained by the low variability of the weight of the bucks, the coefficient of variation being 14.6. In the Common American bucks, the coefficient of variation was nearly twice as great (27.1) and the correlation of buck weight with semen volume 0.204 and significant at the 5 per cent level. For the total population of Toggenburgs and Common Americans, the coefficient of variability was 22.08 and the correlation of buck weight to semen volume 0.227 and highly significant. Correlations between buck weight and concentration of spermatozoa and total spermatozoa were both significant for the total population but not when calculated within the breed, except the one for buck weight and total spermatozoa within the Common Americans.

In the data of Phillips *et al.*[5] with four breeds of sheep, the only significant breed difference in semen characteristics was in total spermatozoa. Data were not presented regarding the weight of the rams used. However, of the breeds used, the Hampshires and Shropshires usually exceed Southdowns and Karakuls in live weight. The average semen volume for the former two breeds was 1.08 cc., while for the two latter ones it was 0.78 cc. Thus, there appears to be agreement as to the effect of size on volume of semen produced among rams and bucks.

Summary and Conclusions

Data are given on semen studies from Toggenburg and Common American bucks, some of them being on observation for three consecutive years.

The characters observed were semen volume, motility, concentration, total number of spermatozoa, and percentage of abnormal spermatozoa.

Semen collection was made at four-week intervals.

Only bucks which had eight or more collections during a twelve-month period have been considered in the data presented.

Averages for all bucks for all seasons for the various characters studied are: semen volume, 0.650 cc.; motility, 1.51; concentration, 2.724 billion spermatozoa per cubic centimeter; total spermatozoa, 1.658 billion; percentage of abnormal spermatozoa, 8.46.

The greatest source of variaion for all of these characters was the individual bucks. The period during the year was an important source of variation in semen volume and concentration. Year-to-year variation was unimportant except for those which were observed for three years, and this effect was on semen volume.

Variation as to season of the year showed semen volume to be greatest in summer and fall, and lowest in spring. Motility was highest in summer and fall, lowest in winter. Concentration of spermatozoa was highest in spring, lowest in fall, while total number of spermatozoa was highest in summer and lowest in spring. The percentage of abnormal spermatozoa was highest in summer and spring and lowest in fall. In general, these characteristics were better in the normal breeding season and poorer in the anestrous period of the does.

Neck and middle-piece abnormalities far exceeded other types.

Correlations showed a negative relationship between volume and concentration, motility and percentage of abnormal spermatozoa, and volume and percentage of abnormal spermatozoa. The relationships between volume and total spermatozoa and concentration and total spermatozoa were positive.

Breed differences between the Toggenburg and Common American bucks were

significant for semen volume, concentration. total spermatozoa, and percentage of abnormal spermatozoa. Some of this difference, so far as semen volume is concerned, may be affected by the buck's weight. Age of the bucks appears to have an influence on sperm motility a.id percentage of abnormal spermatozoa. These two characters bear a significant relationsh.p to one another. For other semen characteristics, there was no significant age effect.

The data agree closely with similar data for rams made at an earlier period.

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The Evaluation of Acid-Fast Allergens on Sensitized Goats

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JOHNE'S DISEASE and tuberculosis are diagnosed in cattle by injecting the appropriate allergen (johnin or tuberculin) into the skin and observing the injection site for a reaction. Unfortunately, these allergens have been shown to contain nonspecific factors which may cause them to elicit reactions when injected into cows sensitized to other than homologous acid-fast bacilli.[1] Such reactions tend to confuse the diagnosis.

Investigations to develop more potent and specific allergens for the diagnosis of Johne's disease and tuberculosis have been under way for a number of years.[2] For these investigations, cattle artificially sensitized with either *Mycobacterium paratuberculosis* or *Mycobacterium tuberculosis* var. *bovis* were tested with the standard allergens (johnin and tuberculin) and with chemically fractionated portions of these allergens.

A total of 64 separate intradermal injections can be made simultaneously by distributing them over the body of a bovine animal, according to a method of distribution planned by Wadley.[3] The reactions resulting from these injections are measured[4] and such measurements are used to make statistical comparisons of the various fractions with each other, and with the parent materials, to determine potency and specificity.[2] Such a method is satisfactory for making such comparisons, except that cattle are expensive to purchase and maintain for evaluating these products. Therefore, experiments were planned to determine if goats could be substituted for cattle as test animals in making such comparisons. It is the purpose of this paper to present the results of these experiments.

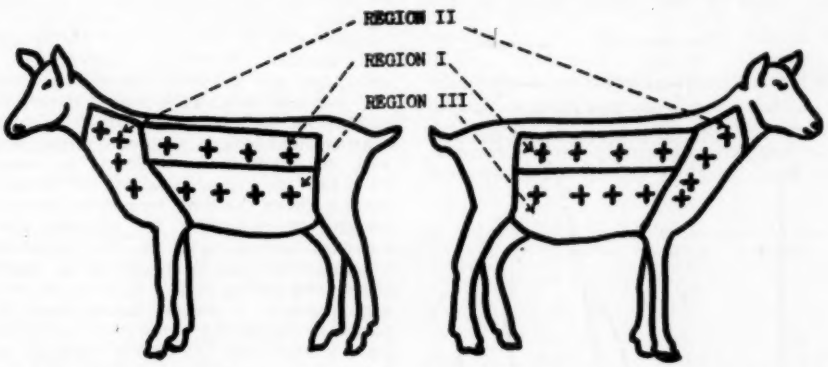

Fig. 1—Location of skin regions on right and left sides of goats. Injection sites in each region = +.

PROCEDURE

Twelve kids of mixed dairy breeding were purchased for the experiment. They were 3 to 5 days of age at the time of purchase and were negative to both intradermal tuberculin and intradermal johnin tests. They were di-

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vided into two groups of 6 goats each and at 6 month of age were sensitized for the experiment. All the goats in one group were sensitized by intraperitoneal injection of 10 ml. of a mineral oil suspension of heat-killed *M. paratuberculosis* prepared by a method similar to that described by Johnson.[5]

Each of the goats in the second group was similarly sensitized with *M. tuberculosis* var. *bovis*.

After administration of the sensitizing dose, an interval of thirty days elapsed before testing began to allow the sensitivity to develop. Three regions were outlined on each side of each animal, based upon observations made in a previous experiment on cattle.[6] Four injection sites about 4 in. apart were selected in each of the three regions on both sides of each animal, making a total of 24 injection sites per animal (fig. 1). Each site was marked by clipping the hair from a small area. After clipping and before making an injection, the skin thickness of each injection site was measured with a dermal thickness gauge.[4]

Three separate series of tests were conducted on the goats at thirty-day intervals. Each test series consisted of three materials injected twice in four concentrations on each goat, with the injections arranged to allow each material to be tested four times on each of the three areas over the 6 animals of each sensitization. The three materials used in test series No. 1 were, standard johnin, standard tuberculin, and tuberculin fraction M-3-193. The three materials used in test series No. 2 were standard johnin, johnin fraction J-3, and johnin fraction J-4. The three materials used in test series No. 3 were standard tuberculin, tuberculin fraction T-10, and tuberculin fraction T-11. Statistical analysis of reactions elicited by M-3-193, J-4, T-10, and T-11 showed them to be significantly more specific than the parent materials when tested on sensitized cattle, while J-3 approached significance. Using saline, a serial dilution technique was employed in making the different concentrations in micrograms of nitrogen per milliliter from an original material (*see* table 1 for an example of this technique as used in test 1).

Each injection was 0.2 ml. in volume. The increase in skin thickness, resulting from the reactions, was measured with the dermal thickness gauge forty-eight hours after injection.

Graphical analysis was used to evaluate the specificity of each material (fig. 2 shows an example of such an analysis). This method consists of plotting the total reaction for each concentration of each material tested against its logarithm of nitrogen concentration. In estimating the specificity factor, the standard test material is given a value of one as its potency on both homologous and heterologous sensitizations and it is tested simultaneously with the fraction to which it is compared. To make the comparison, the horizontal distance between the lines formed by plotting the results obtained with the fraction and the standard test material on the homologously sensitized animals is measured in logarithm units. The antilog of this figure is the homologous potency. A similar measurement is obtained by measuring the distance between the lines plotted from the results obtained on the heterologously sensitized animals. The antilog of this figure is the heterologous potency. The specificity factor is obtained by dividing the homologous potency by the heterologous potency. Such a method is used because it has been shown that practically a linear relationship exists between the logarithms of the concentrations and the size of the resulting reactions. The specificity factor obtained on goats was compared with the factor previously obtained on cattle with the same test material. A more detailed description of the method of analysis can be obtained by consulting papers by Bliss and Marks,[7] Wadley,[8, 9] and Baisden, *et al.*[3]

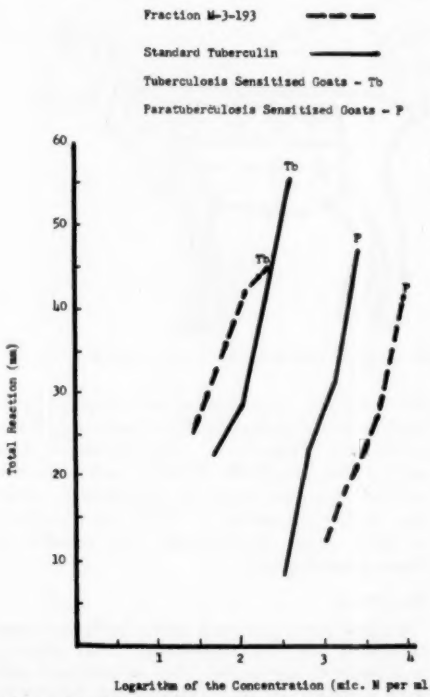

Fig. 2—Graphic comparison of standard tuberculin and fraction M-3-193 on goats.

RESULTS

One *M. paratuberculosis*-sensitized animal died while under test, leaving only 5

animals of that sensitization group. In making a comparison of the results on the two groups, it was decided to subtract one sixth of the total obtained from all the readings obtained on the 6 *M. tuberculosis* var. *bovis*-sensitized animals.

Results of Test Series No. 1.—The micrograms of nitrogen per milliliter of standard tuberculin and tuberculin fraction M-3-193, the logarithm of nitrogen concentration of each testing material, and the adjusted reactions in millimeters are shown in table 1. The johnin readings are not shown, since standard johnin was used to observe the response to this product so that suitable concentrations for injection in the second test could be estimated.

Figure 2, which was prepared from the information shown in table 1, shows the result obtained by plotting the total reaction for each concentration against its logarithm of nitrogen concentration.

After making the necessary calculations from the information plotted in fig. 2, a specificity factor of 8.1 for fraction M-3-193 was obtained. This compared favorably with the value of eight previously obtained on cattle with this fraction.*

Results of Test Series No. 2.—The same method of plotting described previously was used for evaluating the specificity of the materials used in this test and in test 3. Johnin fraction J-4 and J-3 showed specificity factors of 4.4 and 1.25, respectively, on goats. A specificity factor of 3.0 was previously obtained with J-4 on cattle* which is somewhat parallel to the results obtained on goats. However, a specificity factor of 1.25 obtained with J-3 on goats did not compare favorably with a specificity factor of 2.8 obtained on cattle.*

Result of Test Series No. 3.—Tuberculin fractions T-10 and T-11 were found to have specificity factors of 5.4 and 3.3, respectively, on goats. These two materials used at the same concentrations on cattle were found to have specificity factors of 3.8 and 2.9,* respectively, which are roughly parallel to the results obtained on the goats.

*Unpublished data.

DISCUSSION

Since general agreement was obtained on goats and cattle with three tuberculin fractions and one johnin fraction, it is believed that sensitized goats compare favorably with sensitized cattle for testing the specificity of tuberculin and johnin fractions. Although johnin fraction J-3 showed a specificity factor of only 1.25 on goats as compared to 2.8 on cattle, such variation in biological studies may be expected. As previously mentioned, J-3 was the only testing material used which

TABLE 1—Micrograms of Nitrogen per Milliliter, the Logarithm of Nitrogen Concentration of Each Testing Material, and Total Reactions in Millimeters. Forty-Eight-Hour Readings on Goats

	Concentration			
	1	2	3	4
Standard tuberculin on *Mycobacterium tuberculosis* var. *bovis*-sensitized goats				
Micrograms of N/ml.	400.0	200.0	100.0	50.0
Log of the con.	2.6	2.3	2.0	1.7
Reactions (48 hr.)	67.2	51.2	34.4	27.4
Adjusted reactions (48 hr.)*	56.0	42.2	28.0	22.8
Standard tuberculin on *Mycobacterium paratuberculosis*-sensitized goats				
Micrograms of N/ml.	2400.0	1200.0	600.0	300.0
Log of the con.	3.4	3.1	2.8	2.5
Reactions (48 hr.)	47.0	31.0	23.0	8.2
M-3-193 on *M. tuberculosis* var. *bovis*-sensitized goats				
Micrograms of N/ml.	200.0	100.0	50.0	25.0
Log of the con.	2.3	2.0	1.7	1.4
Reactions (48 hr.)	53.8	50.0	38.2	30.6
Adjusted reactions (48 hr.)*	44.9	41.7	31.9	25.5
M-3-193 on *M. paratuberculosis*-sensitized goats				
Micrograms of N/ml.	7700.0	3850.0	1925.0	962.5
Log of the con.	3.9	3.6	3.3	2.99
Reactions (48 hr.)	41.8	26.6	18.6	13.0

*Each of the figures on this line equals five-sixths of the figure just above it, to adjust for the death of a *Mycobacterium paratuberculosis*-sensitized goat.

was not significantly more specific than the parent material when tested on cattle, so this one fraction may not have been a good choice for use in this experiment.

While only 24 simultaneous injections are made on a goat compared with 64 on a bovine animal, the use of goats would result in substantial savings since goats can be bought and maintained for approximately one-sixth the cost of purchasing and maintaining cattle.

SUMMARY

Eleven goats artificially sensitized, 6 with *Mycobacterium tuberculosis* var. *bovis* and 5 with *Mycobacterium paratuberculosis*, were compared with similarly sensitized cattle in the evaluation of chemically fractionated portions of johnin and tuberculin. Measurements of skin reactions resulting from injection of the allergens were compared and the results indicated that goats compare favorably with cattle in evaluating increased specificity of such fractions.

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Dramamine and the Vestibular Apparatus in the Dog

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SINCE THE advent of motor transport, the veterinarian is often requested to advise how to prevent a dog from vomiting every time it is taken for an auto ride.

There is no doubt that accommodation does occur, and fairly rapidly in most cases if the rides are repeated often. There are reports, however, of older dogs that still vomit after numerous, repeated trials.[12]

Many of the drugs used for human beings have been used with questionable success in dogs. The new drug, dramamine, backed by numerous reports of its efficacy for human beings, was logical choice for further detailed and controlled studies in dogs.

Dramamine, a theophyline compound, was originally investigated as an antihistimine drug. Among the patients to whom it was administered was a pregnant woman with urticaria who, in addition, had suffered all her life from car sickness. This patient immediately noticed that the drug was preventing her expected attacks of car sickness. She reported her discovery to the Johns Hopkins' investigator who found it possible to control her symptoms at will. While a placebo failed repeatedly, dramamine gave full protection whenever it was taken a few minutes before she boarded a street car. It was then used on scattered individuals with histories of car or air sickness. The good results prompted a full scale trial in 1948 on a large group of men on an Army transport. Dramamine has been used in doses of 100 to 400 mg. per day as a preventive in human beings, and, even after sea sickness had begun, this dosage gave relief within one hour to 95 per cent affected. The effective human dosage is from 1 to 4 mg. per pound of body weight.

LITERATURE

It was recognized that certain symptoms were referable to similar types of motion whether on land, sea, or in the air. Morton[11] listed the following symptoms in man in the order of their usual occurrence—epigastic awareness, pallor, malaise, nausea, vomiting, sleepiness, yawning, headaches, sweating, abdominal cramps, salivation. It is interesting to note that no matter how violent the illness, all the symptoms disappear rapidly as soon as the causative motion ceases.

McEachern[6] listed the following possible mechanisms of motion sickness—visual stimulation, kinesthetic influences, psychological, vestibular irritation, schism between visual and kinesthetic impressions, vascular factors. They stated that "rapid acceleration and deceleration, especially in the linear plain, is the main factor in the production of motion sickness." Babkin[1] added two causes to the above as visceral displacement and a chemical acidosis in the blood stream.

There have been numerous experiments on man and animals to induce motion sickness experimentally. Groups of Canadian workers did a great deal of work during World War II. No matter how many causes might be listed as contributory, the role of the vestibular apparatus was generally conceded as being primary.

Various animals, besides man, have been found to be susceptible to motion sickness. Dogs, sheep, cows, monkeys, and cats are affected while very young puppies, rabbits, guinea pigs, and most birds are not affected.

The vestibular apparatus consists of the vestibular nucleus in the cerebellum, the vestibular nerve which is a branch of the eighth cranial nerve, and the labyrinth. The labyrinth consists of the auditory and nonauditory part. The auditory part is the cochlea which is connected to the saccule. The nonauditory portion is composed of the three semicircular canals, arranged in the various planes of the head, and two cavities, the urticle and the saccule. The endolymph, which fills the three canals and two cavities, is activated by various motions of the head. These movements of the liquid are then translated to movements of the cilia and otoliths which are in contact with the nerve ending of the vestibular nerve. The impulses are then scattered to the vestibular nucleus in the medulla and cerebellum, to the pons, to the vestibuloocular tract, and to the muscles by way of the spinal motor neurons.[3]

McNally[7] cited many authors from Goltz[5] in 1870 to the present time who have stated that "the labyrinth plays the most important role in motion sickness." "The saccule is not concerned with equilibrium." "The urticle is the most important gravity organ in the labyrinth. It is the end organ for the perception of linear

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acceleration." Sjoberg[17] stated that "motion sickness is the result of stimulation of the semicircular canals and the otolithic apparatus."

It was early noted that any damage to the vestibular apparatus or the labyrinth caused nystagmus. As described by McNally,[8] "Nystagmus is any involuntary to and fro movement of the eyes with a rhythmic quick and slow or undulatory movement in which both components are equal. The movements are usually coördinated bilaterally but may be dissociated or affect only one eye. It may be spontaneous or induced, vestibular, ocular, or central."

Prince[16] in 1920 working on cats was unable to produce any appreciable reduction in the "after nystagmus" by repeated horizontal rotation for twenty days. He concluded that rotation tests give an adequate index to labyrinthine function by measurement of the nystagmus produced.

The principal symptoms that dogs exhibit when sick due to motion are salivation, nausea, and vomiting. Their mucous membranes may become blanched and they may yawn before the salivation starts. They are definitely apprehensive. These symptoms are almost identical with the symptoms in human beings. Dogs will salivate and vomit much more readily than human beings and with much less discomfort. Babkin[1] and Noble[10] have adequately described these factors in their experimental and naturally occurring cases.

TABLE I—Oral Tolerance to Dramamine

Dog	Weight (lb.)	Rate of dosage (mg. per lb. body weight)	Dose (mg.)	Reactions
J	42	5	200	No reaction; good appetie
K	18	5	100	No reaction; good appetie
C	20	10	200	No reaction; ate well
L	75	10	750	No reaction; ate well
B	30	15	450	No reaction; ate well
G	34	15	500	No reaction; ate well*
H	39	20	800	Slightly unsteady gait; ate food, vomited one hour later**
I	12	20	250	No appetite vomited within one hour

*Due to difficulty in administration, two or three tablets were crushed in the mouth. A small vomitus of phlegm and saliva immediately followed. Later, the dog ate well.

**This dog had chronic nephritis.

All animals were offered food ten minutes after dramamine dosing. The maximum tolerated single dose of 15 mg. per pound of body weight is about 50 times the recommended dose (single) for human beings.

The efforts to determine the cause of motion sickness were many and some strove to clarify the type of motion which would give the highest degree of sickness. Others tried to determine the chemical factors in the blood, the blood pressures, the brain waves, the heart action by electrocardiography. Noble[11] found that a swing covering a 14-ft. arc at a 45-degree angle brought about the greatest percentage of vomiting in the dogs tested. He found that linear acceleration and deceleration, with increased rates of speed, increased the incidence of vomiting. Swinging, horizontal, vertical, and angular motion were important in that order. Other motions like "washboard", traverse, "ferris" wheel rolling were also used but showed less effect than swinging.

The majority of all these researches have placed the principal mechanism as being in the vestibular apparatus. Sjoberg[17] found that sections of the eighth nerve or bilateral labyrinthectomy prevented all symptoms of motion sickness. He had noted that deaf mutes who had reactionless labyrinths were entirely immune to motion sickness.

Once the factors of motion sickness were standardized to the point where results could be regularly reproduced, the problem of assaying various drugs could be attacked.[18] Noble[13] developed methods of assaying the relative activity of barbiturates and other drugs. Babkin also found that certain drugs would bring on motion sickness while others, either alone or in combination, would delay or suppress entirely any motion sickness. Noble[12] also worked out some of the factors concerned with the manner in which dogs could accommodate themselves so that their susceptibility decreased.

How to prevent a dog from being affected by motion sickness can be useful to the veterinarian and a blessing to the owner of the dog. Babkin[1] favored a combination of sodium bromine and bulbocapnine. Elliott[4] found that "V-12" (mosidal Abbott) in dose of 3 mg. per pound of bodyweight would adequately protect a dog. Noble, who tested a whole series of barbiturates, in a personal communication wrote, "You will note that many barbiturates are completely effective therapy for dogs." In our own practice, we found that sodium amytal or nembutal, 1½ gr. per 25 lb. of body weight was enough to suppress motion sickness if given one to two hours before motion.

OBJECTIVES

1) To use rotation as a vestibular irritant and to measure the irritation by the resultant nystagmus.

2) To make a limited study of the

pharmacology and toxicology of dramamine in dogs.

3) To determine any vestibular depressant action of dramamine.

4) To determine effect of dramamine on the vomiting center of the dog.

METHODS

1) The 36 dogs used varied in age from 4 months to 16 years and in weight from 10 to 75 lb. They were about equally divided as to sex. They were all healthy dogs except that the older dogs had those chronic conditions which are inevitable in old dogs. The temperament varied from large, placid dogs to very fine and nervous animals.

2) A modification of the Barany, or whirling chair, was used to produce vestibular irritation in dogs. Thirteen dogs were used in trials involving rotation. By mounting a box in the seat of the Barany[9] chair, it was possible to secure the dog in a standing, fixed position while the rotations were carried. The motion was similar to that of a phonograph record on a turntable. After many trials the following procedures were followed in determining the relationship between the speed and number of rotations and the resulting nystagmus in the dogs. The speed of rotation varied roughly from 35 to 45 revolutions per minute. The rotations were carried on as evenly as possible and the onset of nystagmus was computed from a sudden stopping. The duration of nystagmus was estimated by observing the lateral sweep of the eyeballs until such time as the dog showed evidences of being able to voluntarily control the eye movements. This point is usually clear-cut and the error is hardly ever greater than one-half second. The use of a stop watch was imperative. Nystagmus begins as soon as rotation ceases. This type of nystagmus is therefore known as "after nystagmus".[10] The interval is the extent of nystagmus in seconds. Fifty rotations are made clockwise. The extent of nystagmus is noted. Fifty rotations counter clockwise are made and the extent of nystagmus is again noted. The average in seconds is taken as the maximum elictable nystagmus. The dogs were then whirled 15 rotations clockwise and then counter clockwise for four whirls. The duration of nystagmus was recorded after each whirling. The average of the four runs was accepted as being a quantitative representation of the sum total of the vestibular activity, brain conductivity and muscular eye activity. (*See* table 2 for representative data sheet.)

TABLE 2—Dramamine Versus Vestibular Irritation in Dog C (Weight 18 lb., Given 100 mg. Dramamine Orally at 9 a.m.)

Time	Number and direction of rotations	Nystagmus (seconds)	Average nystagmus (seconds)
0	100 R CCW	8.0	12.2
	50 R CW	16.4	
20 min.	15 R CCW	9.0	
	15 R CW	15.5	12.15
	15 R CCW	10.1	
	15 R CW	14.0	
2 hr. 45 min.	15 R CCW	9.5	
	15 R CW	10.0	9.95
	15 R CCW	10.5	
	15 R CW	9.8	
6 hr. 25 min.	15 R CCW	8.6	
	15 R CW	11.2	
	15 R CCW	7.2	9.12
	15 R CW	9.6	
9 hr. 40 min.	15 R CCW	5.0	
	15 R CW	11.0	
	15 R CCW	9.0	9.42
	15 R CW	12.7	
11 hr. 35 min.	15 R CCW	8.6	
	15 R CW	10.9	10.15
	15 R CCW	9.4	
	15 R CW	11.7	

R = rotation. CCW = counter clockwise. CW = clockwise.

TABLE 3—Intravenous Apormorphine Tolerance Test

Dog	Weight (lb.)	Apomorphine (gr. per lb. body weight)	Apomorphine total dose in gr.	Results
T	21	0.000025	0.00052	No vomiting; drank milk
V	32	0.000025	0.0008	No vomiting
L	75	0.000025	0.001875	No vomiting
W	14	0.00005	0.0007	Vomited in three minutes
G	34	0.0001	0.0034	Vomited in three minutes
I	12	0.0001	0.0012	Vomited in two minutes

3) Twelve dogs were given oral doses of dramamine while 3 were given intravenous doses. The oral administration was given at various times in relation to feeding and seemed to make no difference. When the tablets were crushed in the mouth, there was excessive salivation until the toxic level was reached. Within nontoxic ranges no other discomfort nor interference was noted with appetite or bowel function.

Single doses were given to find the maximum nontoxic level. Daily doses were given to observe cumulative toxic reactions. For intravenous use, the dramamine was dissolved in

heated saline and dextrose solutions. (*See* table 1.)

4) To assay the action of dramamine against motion sickness, an attempt was made to inhibit or counteract the action of apomorphine hydrochloride by first dosing with moderate doses of dramamine. The apomorphine was then administered either subcutaneously or intravenously. One of the immediate problems arising from the stimulation of the vomiting center was to find out the minimum dose of apomorphine that would cause vomiting. By repeated trials and by drug weight to animal weight relationships, the proper dosage was determined.

TABLE 4—Combined Apormorphine Injection and Whirling

Dog	Weight (lb.)	Apomorphine (gr. per lb. body weight)	Total dose in gr.	Time of vomiting	Effect of 200 rotations
X	10	0.0001	0.001	....	Slight salivation
W	10	0.0001	0.001	....	None
I	11	0.00015	0.00165	4:45	.....
G	30	0.00015	0.0045	....	No effect
L	75	0.0002	0.015	4:45	.....
Y	34	0.0002	0.0064	4:45	.....

Doses of apomorphine from 0.00005 gr. to 0.0001 gr. per pound of body weight will cause vomiting within three minutes. There is one, or at the most two vomitions, the animals show no distress, and are ready to eat within a few minutes after emesis. (*See* table 3.)

Dramamine at the rate of 5 mg. per pound of body weight was given to the dogs at 2:00 p.m. The dogs were then fed their usual meal at 3:00 p.m. The injections of apomorphine sulfate were given subcutaneously from 3:35 p.m. to 3:38 p.m. The concentration of apomorphine was .0002 gr. per pound of body weight.

Dramamine in moderate to high doses did not inhibit the action of minute doses of apomorphine, which caused 5 out of 6 dogs to vomit. (*See* table 5.)

5) Since vomiting due to motion is known to be a complex mechanism, an attempt was made to induce vomiting in dogs with whirling after they had maximum nonvomiting doses of apomorphine to lower the vomiting threshold. Animals not vomiting within fifteen minutes after apomorphine injection were given 200 rotations, alternating the direction at the end of 50 rotations. The vestibular irritation as manifested by the extent of nystagmus was also recorded before and after the apomorphine. (*See* table 4.)

The object of this experiment was to graduate the subcutaneous dose of apomorphine so low that there would be several animals receiving maximum nonvomiting doses of apomorphine. These were then to be whirled for 200 rotations to see whether vomiting could be induced.

The dogs were fed at 3:10 p.m. and then whirled to record the duration of nystagmus before apomorphine injection. They were then fed again at 4:25 p.m. The apomorphine was injected at 4:30 p.m.

Dogs X, W, and G were whirled, since they did not vomit within fifteen minutes, after the subcutaneous injection of the apomorphine. The results as far as vomiting, or the duration of nystagmus, were not significantly altered.

RESULTS

Pharmacology and Toxicology of Dramamine in Dogs.—Dogs were dosed daily in quantities varying from 1 mg. per pound of body weight to 100 mg. per pound of body weight. Single doses were well tolerated up to 15 mg. per pound of body weight. One large dog took a single dose of 750 mg. without any untoward effects. Any dose from 20 mg. and up per pound of body weight will cause vomiting in at least half the dogs. Fifteen dogs were used in the various feeding and injection studies. Table 1 represents a typical data sheet.

Daily doses of 4 mg. per pound of body weight were well tolerated for five days. When this dose was doubled, the dogs began to vomit and to have diarrhea after three days (2 out of 3 cases). No hematological studies were performed.

Dosages from 20 mg. to 40 mg. per pound of body weight cause vomiting. As the dose increases, the rate of vomiting increases from 50 per cent to almost 100 per cent. One hundred mg. per pound of body

TABLE 5—Dramamine Versus Minimum Doses of Apormorphine Causing Vomiting

Dog	Weight (lb.)	Total dose dramine in mg.	Total dose Apomorphine in gr.	Results
AB	25	125	0.005	Vomited at 3.50
G	30	150	0.006	Vomited at 3.40
B	30	150	0.006	No vomiting; ate at 3.50
AC	35	175	0.007	Vomited at 3:40
AD	40	200	0.008	Vomited at 3:42
L	70	350	0.014	Vomited at 3:52

weight causes severe reaction with prostration, vomiting, dilation of the pupils, nystagmus, and death within thirty minutes.

Intravenous 1 per cent dramamine in isotonic saline, and 5 per cent dextrose was used in dosages of 4, 5, and 10 mg. per pound of body weight. With 4 and 5 mg. per pound of body weight, there were slight and transient symptoms of trembling, confusion, and some muscular incoördination. Recovery was rapid, and the dogs had a good appetite within three hours. On a dosage of 10 mg. per pound of body weight, there was severe prostration, tremors, hyperesthesia, and incoördination to the point of loss of balance. The effect of the drug in this dosage was much more lasting and the effects were not completely removed after twenty-four hours.

With moderate doses there were no signs of abnormal or significant changes in muscular control, blood pressure, pulse, or respiration. There were no signs of flatulence, colic, bloating, or nausea. There was no loss of weight in animals that were under trial for two months. Variations in age, sex or temperament did not seem to affect the use of dramamine.

Stimulation of the Vestibular Apparatus.—Rotation in the Barany chair was very satisfactory in getting marked response in the form of active lateral sweeps of nystagmus in the eyes. The maximum nystagmus could be elicited after approximately 50 rotations. Of the 14 dogs that were tested, the maximum nystagmus averaged 14.875 seconds. The spread was from 8.12 seconds to 18.5 seconds. The average duration of nystagmus after four sets of 15 rotations was 11.4 seconds.

Motion sickness as manifested by vomiting or severe nausea was never brought about by rotation. Continuous rotation for twenty minutes, alteration in the direction of rotation, repetitions of rotation from time to time, and increasing the speed of rotation were of no avail.

Ability of Dramamine to Suppress Nystagmus.—Six dogs were used in trials to see whether dramamine would inhibit or suppress vestibular irritation and/or nystagmus. The whirling trials were made before and after the dosing of the dramamine. (*See* table 2.) Trials were first made using oral dosing and the whirling was continued for periods up to 11.5 hours. In order to be sure that a maximum absorption of the drug had occurred, a trial was made using intravenous dosing of dramamine. In neither instance was there any significant change in the nystagmus resulting from standard rotations.

Ability of Dramamine to Suppress Vomiting.—To evaluate the ability of dramamine to counteract the stimulation of the vomiting center by apomorphine, it was imperative to ascertain the least amount of the apomorphine needed to induce vomiting in about 80 per cent of the dogs. Apormorphine tolerance tests were performed on 16 dogs. Table 3 represents one such trial using apomorphine intravenously. This experiment was interesting since it revealed that the recommended dosages in the veterinary and human literature were in most instances too high. Most texts recommend from 1/40 to 1/60 of a grain of apomorphine. Our findings are that 0.0002 gr. per pound of body weight will cause vomiting in more than 75 per cent of the dogs and 0.00025 will give 100 per cent emesis. We therefore recommend this dose that varies from 1/500 to 1/50 of a grain. This means that most dogs have been overdosed almost ten times in ordinary practice. If given intravenously, the dosage, can be further reduced by halving the dose.[9]

Seven dogs were given dosages of apomorphine varying from 0.0001 gr. to 0.0002 gr. per pound of body weight. Of these, 3 vomited. The remaining 4 dogs were rotated from 200 rotations (alternating the direction after every 50 rotations) to see whether the apomorphine would lower the threshold enough so that the combined use of apomorphine and whirling would bring about vomiting. Not one of the dogs showed any effect from the combined action. (*See* table 4.)

Doses of 10 mg. of dramamine per pound of body weight were given to 6 dogs to see whether they would counteract fairly large doses of subcutaneously injected apomorphine. Every dog vomited in this trial. Table 5 gives the results of using dramamine at the rate of 5 mg. per pound of body weight against a minimum dose of apomorphine. Five, out of the 6 dogs tested, vomited. These results preclude any inhibitory effect from dramamine.

SUMMARY

1) Dramamine is well tolerated orally in dogs up to 15 mg. per pound of body weight in single doses and up to 4 mg. per

pound of body weight in daily doses up to five days.

2) Doses above 20 mg. per pound of body weight, orally, will result in vomiting within ten minutes. Doses above 40 mg. per pound of body weight may be considered toxic to dogs.

3) Intravenous doses of dramamine of 4 to 5 mg. per pound of body weight can be tolerated. Doses higher than this are definitely toxic.

4) Vestibular activity was not depressed by dramamine.

5) Apomorphine hydrochloride in doses of 0.00025 grains per pound of body weight is enough to cause vomiting in 100 per cent of the dogs. This is about a tenth of the usual recommended dosage.

6) Vestibular irritation by rotation does not cause motion sickness in dogs.

7) Vestibular irritation by rotation does not lower the vomiting threshold.

8) Dramamine does not antagonize or neutralize the effect of emetic doses of apomorphine in dogs.

9) Clinical trials on dramine for its effect on car sickness were not performed.

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Canine Penicillin Blood Levels Obtained with Parenteral Benzethacil

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SUSTAINED PENICILLIN blood levels, because of differences in environmental sanitation and patient coöperation, are even more desirable in veterinary medicine than in human therapy; too, the cost and inconvenience of repeated doses of any of the usual penicillin salts quickly may exceed the economic limit.

A new repository form of penicillin, N,N'-dibenzylethylenediamine dipenicillin G, or benzethacil,* has been described in detail in the literature.[1, 2, 3] After a single dose of 300,000 units in aqueous suspension given intramuscularly in human beings, demonstrable penicillin blood levels have been obtained from seven to eleven days.[3] Prolonged levels also have been demonstrated in rabbits.

To determine the response elicited by benzethacil in normal dogs, a single intramuscular dose of 300,000 units in aqueous suspension was used. Additional dogs were given potassium penicillin G and procaine penicillin G, similar in amount and preparation, to compare results.

The first group of dogs were cross-bred animals varying from 8 months to 2 years of age, weighing from 35 to 50 lb. Blood samples were drawn without coagulant into sterile test tubes at the postinjection times indicated and stored at the usual refrigerator temperatures; the serum content of penicillin was determined by the microbiological cup plate method with Sarcina lutea as the test organism.

A second group of dogs, Springer Spaniels, weighing from 50 to 60 lbs., used for breeding by a nearby kennel, were made available and the same injection and bleeding program was repeated. The work was discontinued after eight days because the owner objected to further bleeding of the animals.

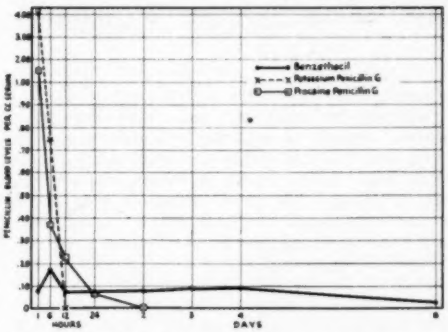

Fig. 1—Penicillin blood levels per cubic centimeter of serum.

The maintenance of sustained, effective blood levels is a primary problem in the treatment of any animal disease in which penicillin therapy is indicated. Attempts to accomplish this end have been directed toward the development of less soluble penicillin salts and by the admixture of absorption-retarding materials such as wax, aluminum monostearate, etc. From the

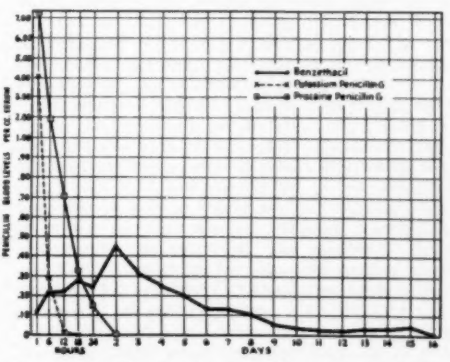

Fig. 2—Penicillin blood levels per cubic centimeter of serum.

From the Department of Veterinary Service, Wyeth, Inc., Philadelphia, Pa.

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*Supplied by Wyeth, Inc.

references quoted and the data here presented, benzethacil appears to be the penicillin salt of choice for the purpose of maintaining prolonged blood levels.

SUMMARY

The administration of a single, intramuscular dose of 300,000 units of benzethacil (N,N'-dibenzylethylenediamine dipenicillin G) to medium-sized dogs resulted in a blood serum-penicillin level usually considered effective for from eight to fifteen days. Similar injections of potassium penicillin G resulted in serum levels for twelve hours and of procaine penicilin G for twenty-four hours.

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Feline Pneumonitis. I. Immunization Studies in Kittens

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FELINE PNEUMONITIS is now recognized as being of considerable importance in feline practice. While the uncomplicated infection seldom terminates fatally except in young kittens, nevertheless, it frequently debilitates older animals to such an extent that complete clinical recovery is greatly prolonged and relapses commonly occur. Furthermore, because of its extreme communicability, this disease constitutes a major problem in pet hospital management.

Apart from the fact that feline pneumonitis has been found to exist simultaneously with panleucopenia in the same animal,[1] little is known regarding its interrelationships, if any, with other feline conditions. Whereas the experimental infection is relatively mild, the severity of the clinical condition from which the virus of feline pneumonitis has been isolated varies within wide extremes. This rather suggests that other factors, either of primary or secondary importance, are involved.

In general, a poor immune response is produced, either by natural or artificial means, to the agents of the psittacosis-lymphogranuloma - pneumonitis group of which the virus of feline pneumonitis *(Miyagawanella felis)* is a member. However, during the course of his studies on the latter virus, Baker[2] found that, in the case of the few cats tested, those which recovered clinically could not be reinfected by intranasal challenge with infectious material. Furthermore, he succeeded in immunizing mice against experimental infection by the inoculation of viable virus intraperitoneally.

In the light of these findings, it was considered advisable to investigate the immunogenic potential of the feline pneumonitis virus in greater detail in the event that vaccination might produce a satisfactory immunity, or at least modify the severity of the infection. Accordingly, since the disease is most severe in young animals, an investigation was undertaken to determine the protective value of vaccines in kittens against the experimental disease. Evaluation of each vaccine was attempted on the basis of the serum antibody levels elicited in response thereto, and on the clinical response of the vaccinated animals to experimental challenge.

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MATERIALS AND METHODS

Virus.—The original strain of feline pneumonitis virus was used throughout in this study. It was provided, in the form of frozen, infected mouse lung from the thirty-eighth serial passage, by Dr. James A. Baker (New York State Veterinary College). A second culture of the same strain which had been adapted to grow in the allantoic sac of chicken embryos[3] was obtained from Dr. Dorothy Hamre of the Squibb Institute, Rahway, N. J. Growth of the mouse lung-adapted virus was readily adapted to yolk sac, and it was propagated in this medium and in mouse lung, while the allantoic sac-adapted virus was maintained by allantoic passage. Suspensions were prepared by grinding tissues from infected embryos and mice in either a glass tissue grinder or a Waring blendor with or without a small amount of suspending fluid. Fluids routinely employed for this purpose were infusion broth, unbuffered saline, saline buffered at pH 7.2 with 4 parts of 0.15 molar phosphate buffer per hundred, and 0.1 molar neutral phosphate buffer. The material was then made up to a 10 per cent suspension with the same suspending medium as used during the grinding. Suspensions used as inoculum were prepared usually by volume, whereas those to be titrated or used for challenge purposes were prepared on an accurate weight basis. In the case of the challenge material, sterile skimmilk was used as the suspending fluid.

Suspensions and fluids to be inoculated were filmed and stained by Macchiavello's method[4] and examined microscopically to ensure that elementary bodies were present. Infectious material was held for periods up to one week in duration at 4 C. or under dry ice refrigeration, if longer periods ensued between harvesting and use of the material. The challenge virus was shell frozen in hermetically sealed vials and held for a period up to one year in duration under dry ice refrigeration.

Titration of Virus.—Titrations were conducted in albino Swiss mice weighing between 15 and 20 Gm. each. In titrating, 0.05 cc. of serial tenfold dilutions of virus were inoculated intra-

nasally while the mice were under light ether anesthesia, using 5 mice for each dilution. The presence of pneumonia on autopsy seven days subsequent to inoculation was taken as the criterion for infection, with or without the demonstration of elementary bodies. The virus content of titrated material was expressed in terms of infective dose$_{50}$ (ID_{50}) according to the method of Reed and Muench.[5]

IMMUNIZATION PROCEDURES

Vaccine Production.—Several harvestings of infected yolk sacs from the sixteenth to the twentieth serial egg passage were pooled and minced in a Waring blendor without diluent. Sufficient 0.1 molar neutral phosphate buffer was then added to make a 10 per cent suspension by weight. Following titration, portions of this master suspension were removed and treated as described below in the preparation of the yolk sac vaccines. Mouse lungs of the sixty-second serial passage, and allantoic fluid from the eighty-first consecutive allantoic egg passage were also used in the preparation of vaccines.

Crude Yolk Sac Virus.—A portion of the yolk sac suspension, which gave an ID_{50} titration value of 3.5×10^{-4}, was dispensed in 2-cc. amounts in lyophile vials, shell frozen, and dried under vacuum. The vials were then hermetically sealed.

Concentrated, Partially Purified, Yolk Sac Virus.—The supernatant fluid obtained by centrifuging a portion of the master suspension at 2,500 revolutions per minute (r.p.m.) for fifteen minutes was recentrifuged at 25,000 r.p.m. in a Sharples centrifuge, using a clarifier bowl. The sediment, which was found to consist largely of elementary bodies, was removed and resuspended in a volume of 0.1 molar neutral phosphate buffer equal to one-tenth that of the original yolk sac suspension. The suspension was then dispensed and lyophilized as previously described.

Purified Yolk Sac Virus.—A mass of elementary bodies relatively free of extraneous material was obtained from a portion of the master yolk sac suspension by precipitating out the soluble yolk sac fractions with ammonium sulphate and recovering the virus by differential centrifugation. The recovered virus was then suspended in a volume of 0.1 molar neutral phosphate buffer equal to one-tenth that of the yolk sac suspension from which the elementary bodies were recovered. The suspension was stored in this form.

Irradiated Purified Yolk Sac Virus.—A suspension of elementary bodies recovered from infected yolk sac as previously described was subjected to ultraviolet irradiation in a closed cabinet for thirty minutes. The suspension was exposed as a film 5 mm. in depth in a flat-bottomed glass container at a distance of 8.1± cm. from the source of the rays. The material was then stored in this form.

Crystal Violet-Treated Purified Yolk Sac Virus.—To a 50-cc. portion of the elementary body suspension, 2 cc. of a 1 per cent aqueous solution of crystal violet was added. The mixture was thoroughly shaken and incubated at 37 C. for ten days.

Allantoic Fluid Virus.—After standing overnight at 4 C. to permit settling of the cellular debris, the supernatant material of a volume of undiluted infected allantoic fluid was dispensed and lyophilized as described. The prelyophilization ID_{50} titer was 5.6×10^{-3}.

Crude Mouse Lung Virus.—A 10 per cent suspension was prepared by shaking coarsely chopped lungs with Pyrex fragments in extract broth. It was then dispensed and lyophilized according to the procedure already described.

All vaccines were tested for viability by nasal inoculation into mice immediately following preparation and then stored at 4 C. until used. Unused portions were returned to storage if they were to be required later.

Vaccination and Challenge of Kittens.—Kittens 6 to 10 weeks of age were given a single subcutaneous injection of 2 cc. of vaccine. Immediately thereafter, the animals were isolated and observations made daily for evidence of infection. Five weeks following vaccination each kitten was challenged, while under light ether anesthesia, with 100 mouse ID_{50} of virus given intranasally. The same amount of virus was administered to 7 unvaccinated kittens which served as controls. The vaccinated and control animals were observed closely for an ensuing period of one month, at the end of which time attempts were made to isolate the virus from ocular and nasal washings. The animals were then destroyed and in cases where the agent had not been recovered, attempts were made to isolate it from the turbinate bones. Results are shown in table 1.

SEROLOGICAL PROCEDURES

The complement-fixation test, because of its highly successful adaptation to studies on virus diseases, and particularly those due to members of the psittacosis-lymphogranuloma-pneumonitis group of agents, was selected as a possible method of evaluating each vaccine on the basis of the antibody response elicited thereto in kittens.

Antigens.—In addition to crude suspensions of infected yolk sac and mouse lung, the high speed sediment from infected yolk sac suspension, suspensions of infected chorio-allantois, and allantoic fluid containing a heavy concentration of elementary bodies were also tested for their suitability as complement-fixing antigens. However, with the exception of the suspensions of infected yolk sac and mouse lung, all were either weakly or nonantigenic. While both of these preparations were equally satis-

factory, the mouse lung antigen was selected for use in the test inasmuch as the majority of the vaccines were prepared from infected yolk sac. Thus, the necessity for testing the serums against normal yolk sac was eliminated.

Mice infected by intranasal inoculation of virus were destroyed when moribund and, after irrigating the lungs to flush out static blood, the consolidated portions thereof were harvested, weighed, and placed in nine volumes of extract broth. The suspension prepared therefrom by shaking with Pyrex fragments was centrifuged at 1,500 r.p.m. for ten minutes to sediment coarse particles. The supernatant material which contained a heavy concentration of elementary bodies relatively free from tissue debris constituted the antigen. Storage was maintained under dry ice refrigeration and the antigen was used over a period of several weeks.

Cat Serums.—Blood samples were obtained by cardiac puncture from each kitten prior to and five weeks following vaccination, and again at the time of destruction one month following challenge. The control animals were bled at the time of infection and one month subsequently. After clot shrinkage and centrifugation, the serums were withdrawn and held in the frozen state.

Test Procedure.—Serial two fold dilutions of each serum specimen were made in physiological saline and 0.2 cc. of each dilution was added to Wassermann serological tubes that had been thoroughly washed and acid rinsed. Positive and negative control serums similarly diluted were included in each test. Two units of antigen in saline contained in a volume of 0.2 cc. were then added to each tube, followed by the addition of 2 units of complement in saline in a volume of 0.5 cc. Each serum specimen was also tested for anticomplementary activity in the absence of antigen. The three control tubes contained, in addition to 2 units of antigen, 2, 1, and 0.5 units respectively of complement in a volume of 0.5 cc. The test mixtures were incubated for two hours in a water bath at 37 C. To each tube was then added 1 cc. of a sensitized cell mixture consisting of equal parts of a 2 per cent sheep red blood cell suspension and 2 units of hemolysin in saline, mixed from twenty to thirty minutes previously. The tubes were then shaken and

TABLE I—Clinical Response of Vaccinated and Control Kittens to Active Virus and Serum Antibody Titers Evoked in Response to Vaccination and to Infection

Vaccine	Number of kittens vaccinated	Number resistant to challenge	Number yielding virus one month post-challenge	Serums tested	Distribution of antibody titers <1-4	1-8	1-16	1-32	1-64	1-128	Ave. titer of group
Crude yolk sac** virus	5	4	2	Prevaccination	5						<1-4
				Postvaccination			1			4*	1-105
				Postchallenge					4*	1	1-77
Concentrated partially purified yolk sac** virus	6	6	5	Prevaccination	6						<1-4
				Postvaccination		1	2	1	1	1	1-44
				Postchallenge		1		1	3	1	1-50
Purified yolk sac** virus	5	3	4	Prevaccination	5						<1-4
				Postvaccination	1*	2	2*				1-9
				Postchallenge			1	1		3*	1-85
Irradiated purified*** yolk sac virus	6	4	4	Prevaccination	6						<1-4
				Postvaccination	1	1	4*				1-12
				Postchallenge			1	3*		2*	1-60
Crystal violet*** treated purified yolk sac virus	4	3	3	Prevaccination	4						<1-4
				Postvaccination	3			1*			1-8
				Postchallenge		1	1	1		1*	1-46
Allantoic** fluid virus	3	1	2	Prevaccination	3						<1-4
				Postvaccination			2*	1*			1-21
				Postchallenge				3*			1-32
Crude mouse lung** virus	5	5	0	Prevaccination	5						<1-4
				Postvaccination	2	1	1	1			1-11
				Postchallenge	2	1	2				1-8
Control Kittens	number inoculated	number infected	number yielding virus one month post-inoculation								
	7	7	7	Preinfection	7						<1-4
				Postinfection		2	3	2			1-18

*Serum titer of kitten(s) in which clinical infection developed following challenge. **Viable vaccine. ***Nonviable vaccine.

reincubated. Readings were made when hemolysis was complete in the negative serum control series and in the tube containing 2 units of complement, and partially so in the one containing 1 unit. Readings were recorded on the basis of the relative concentration of the red blood cells in suspension in each tube. Complete, rather than partial, fixation was taken as the endpoint. The titers shown in table 1 refer to the initial dilution of the serum before the addition of the other reagents.

RESULTS

The results are shown in table 1.

The great majority of the vaccinated kittens failed to manifest clinical evidence of feline pneumonitis following experimental challenge although virus was present in the respiratory tract of most for at least one month thereafter. In general, the vaccines containing active virus protected a greater proportion of the animals against challenge than did those containing the inactivated agent. Characteristic symptoms of the disease which were displayed by several of the vaccinated kittens following challenge were mild as compared with those produced in all the control animals. In no case did clinical infection result from vaccination with the living agent.

An antibody response in terms of the complement-fixing titer of the serum was produced in the majority of the vaccinated kittens which, in some cases, exceeded that of the experimentally infected controls. However, lack of correlation was noted in many instances between the antibody titers resulting from vaccination and clinical resistance to challenge. Low titers which were produced in response to vaccination were found, in the majority of cases, to have increased following challenge.

DISCUSSION

The fact that the majority of the vaccinated kittens withstood challenge with the same amount of virus which caused active infection in all cases among the control animals is evidence that vaccination against feline pneumonitis, with either dead or living virus, stimulates a degree of resistance to the fully virulent virus. The titer of the majority of the vaccinated kittens was found to have increased beyond the postvaccination level following challenge, which suggests that the virulent virus produced an anamnestic reaction in such animals. Should this occur in vaccinated kittens as a result of natural exposure, it is a most fortuitous circumstance inasmuch as it could, ostensibly, prolong the artificially induced immunity indefinitely, providing that exposures occurred at sufficiently frequent intervals.

The absence of a consistent parallel which was noted between the antibody titers resulting from vaccination and resistance to clinical infection might be interpreted as evidence that immunity in feline pneumonitis is not necessarily related to the presence of complement-fixing antibodies. However, inasmuch as challenge was followed by the development of appreciable titers, even in animals which exhibited little or no serological response to vaccination, vaccination might be regarded as inducing a conditioning of the antibody-producing mechanism of the body so that, under the stimulus of challenge, protective antibodies were readily produced in a further manifestation of the anamnestic reaction mentioned above. Failure to develop antibodies at a sufficiently rapid rate to be of any protective value could account for the coexistence of relatively high serum titers and clinical infection which was demonstrated in a number of instances in this study. On the other hand, it is possible that immunity in feline pneumonitis is of the tissue, rather than the humoral type. The correlation which was observed in some cases in this study between antibody titer and resistance would, therefore, be merely coincidental. However, it must be emphasized that the kittens which were considered as having failed to resist challenge exhibited relatively mild, although characteristic, symptoms of the disease as compared with the controls. Since the only utilizable criterion for the recognition of the disease was clinical evidence, it is possible, although rather unlikely, that manifestations of a nonspecific nature in the case of the vaccinated kittens mentioned above were mistaken for symptoms of feline pneumonitis.

Contrary to expectations, the yolk sac vaccines containing high concentrations of virus failed to stimulate comparable titers to those elicited by the crude infected yolk sac. However, the virus content of the purified and concentrated yolk sac vaccines was not determined by mouse titration. It is, therefore, possible that although these

vaccines contained a much greater concentration of virus than the crude yolk sac vaccine, it was largely in the inactivated state. The same objection would not apply to the allantoic fluid and mouse lung vaccines, however, neither of which was subjected to procedures destructive to the virus. Nevertheless, the antibody response to the latter preparation was of the same order as that evoked by the preparations consisting of the purified yolk sac virus. These observations suggest that the antigenicity of the vaccines used in this study is not primarily dependent on their virus content. It was noted that a close parallel existed between the antigenicity of the vaccine and the toxin content of the material from which it was prepared. It is suggested, therefore, that either the labile toxic substance which is elaborated in large amounts by the feline pneumonitis virus when grown in yolk sac[6] and to a lesser extent in the allantoic cavity,[3] or an antigenic component present quantitatively with it, is the factor largely responsible for the antigenicity of the feline pneumonitis virus. It is difficult, however, to account for the fact that the mouse lung which evoked a poor antibody response proved to be a highly sensitive antigen in serologic tests.

SUMMARY

Of 34 kittens vaccinated with either active or inactivated feline pneumonitis virus, 26 failed to develop observable symptoms of the disease following challenge with viable virus administered intranasally, whereas the controls in all instances developed typical clinical infection. The 8 remaining vaccinated animals displayed characteristic, although relatively mild, symptoms of the disease as compared with the control animals.

The crude yolk sac virus vaccine would appear to be the one of choice, mainly because of simplicity in preparation.

Inasmuch as the most antigenic of the vaccines were prepared from materials which contained considerable amounts of toxin, indications are that complement-fixing antibodies in high titer to the virus are essentially antitoxic in nature. However, the significance of these in immunity to feline pneumonitis is not clear.

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The Influence of Litter on the Control of Salmonella Infections in Chicks

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Kennard and Chamberlin[1, 2] reported a 12 per cent reduction in mortality of chickens reared on old built-up litter. Using over 1,700 chickens they observed a total mortality of 17 per cent among chickens on fresh litter as compared with a mortality of 5 per cent among chickens on old built-up litter. They also observed that chickens reared on old built-up litter grew more rapidly on an all vegetable diet that was deficient in the animal protein factor than did those on litter that was frequently changed. Kennard, Bethke and Chamberlin[3] observed higher hatchability of eggs from hens fed an all vegetable diet and kept on built-up litter as compared to eggs from hens on litter that was frequently changed. The work of Halbrook, Winter and Sutton[4] confirmed this effect on growth and the results of Schlamb and Winter[5] confirmed the work on hatchability. Halbrook, Sutton and Winter[6] also demonstrated that the Vitamin B^{12} content of built-up litter used for one year averaged 261 $\mu\mu$g. per gram of litter, that of new cob litter only 1 $\mu\mu$g. per gram.

Much of the work that has been reported on built-up litter has concerned the nutritional point of view and controlled experiments using infectious agents have not been reported. Therefore, a need for the study of certain pathogenic bacteria and their relationship to the litter and to the reduction of mortality of chickens on such litter becomes apparent.

Any of the materials that are commonly used for floor litter such as ground corn cobs, peanut hulls, peat moss, chopped straw, and wood shavings can be employed in the starting of built-up litter. Four requirements for the proper functioning of built-up litter are: (1) Depth—litter should be 6 to 12 in. deep in order to provide the necessary reservoir for absorption of fresh droppings and for various chemical and biological activities; (2) Stirring—in order to keep the litter in a fully absorbent condition, it should be stirred whenever caking begins to occur. This will also remove the droppings from the surface and permit the various reactions to take place which result in the breakdown of such materials; (3) Moisture—the various chemical and biological activities require some moisture. This is usually provided by the droppings and from the watering devices; (4) Heat—the temperature necessary for the reactions to take place in the litter needs no special attention as there is usually enough heat provided by changes taking place in the litter and by the birds; only during very prolonged cold weather would any additional heat be necessary.

With mature birds, the litter begins to build up in about eight weeks, but the litter should be in use five to six months to obtain the various nutritional values necessary for breeding flocks and for the production of eggs of good hatchability.

Experimental

Salmonella Pullorum.—The strain of *Salmonella pullorum*, 129, was obtained from the Veterinary Laboratory, Department of Bacteriology, at The Ohio State University and had been isolated from a chick during an outbreak of the naturally occurring disease. In order to establish the pathogenicity of this strain of the organism, 150 healthy 24-hour old New Hampshire chicks were given 0.25 ml. of a saline suspension. This was done by inserting a pipette into the mouth and administering the organisms directly into the oral cavity. Twenty-five hundredths milliliter of the suspension contained 250,000 organisms.

These chicks, 30 per pen, were then placed on litter, or on a wire floor as follows: pen 1, new cob litter; pen 2, new cob litter; pen 3, old built-up litter; pen 4, old built-up litter plus lime; pen 5, wire.

All pens were 5 ft. by 7 ft. The temperature was maintained at 38 C. the first week and was gradually dropped to 35 C. by the end of the third week.

A four-fold purpose was accomplished in this procedure: (1) The virulence of the organism was determined; (2) The litter became contaminated with the organisms; (3) The numbers and the length of time that the organisms persisted in the various types of litter was studied; and (4) The infectivity for day-old chicks placed on this contaminated litter was determined.

In trial 1, which was started in November,

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30 chickens were placed in each of the five pens. The 1-day-old chicks were given 250,000 *S. pullorum* (strain 129 organisms in 0.25 ml. orally and the overall mortality was 80 per cent, table 1). In this trial, the litter played no role in the course of the disease.

TABLE 1—Virulence of Salmonella Pullorum (129) for Chicks on Various Types of Litter

Type of Litter	Mortality during 21 days Trial 1 (%)	Trial 2 (%)
Pen 1, new cob	74	80
Pen 2, new cob	70	74
Pen 3, old built-up litter	84	87
Pen 4, old built-up + lime	86	77
Wire	86	100

24-hour-old New Hampshire chicks fed 0.25 ml. of a 24-hour culture (0.25 contained 250,000 organisms); 150 chicks in each trial.

As stated before, one of the aims was to contaminate the litter with *S. pullorum* in an attempt to determine if there was a difference in the persistence of this organism in new cob and in old built-up litter.

The number of Salmonella organisms per gram of litter was estimated by the following procedure. Twenty small random samples were obtained from various parts of the surface litter in each pen. These were mixed together and then a 10-Gm. sample was placed in 90 ml. of distilled water. This dilution was then shaken manually in dilution bottle at intervals for thirty minutes before any further dilutions and plates were made. Shigella-Salmonella (Bacto-SS) agar plates were partially dried either by placing them in an oven at 45 C. for five hours or at 37 C. for twenty-four hours. This drying procedure permitted the development of discrete colonies on the surface so that their colonial characteristics could be observed and representative colonies could be picked and inoculated into Kligler's iron agar and other differential culture mediums. The use of unglazed porcelain lids on the Petri dishes also permitted one to incubate these plates in the upright position. On this medium, lactose-positive organisms are inhibited and the Salmonella colonies appear as small colorless colonies after twenty-four hours of incubation at 37 C. Table 2 gives the estimation of the survival of *S. pullorum* in the various types of litter in the first trial.

TABLE 2—Plate Counts on SS Agar for the Persistence of Salmonella Pullorum in Litter (Trial 1)

Type of litter	Number of organisms per gram after 21 days	32	48	56	66
Pen 1, new cob	225,000	175,000	0*	0*	0
Pen 2, new cob	200,000	120,000	0*	0*	0
Pen 3, old built-up	0	0	0	0	0
Pen 4, old built-up + lime	0	0	0	0	0

*A few organisms were recovered when the sample was placed in Selenite Broth and incubated 24 hours before plating on SS agar.

After twenty-one days, the chicks that survived were removed (table 1), the feed and water trays were cleaned and sterilized, but the litter was not disturbed. Other 24-hour-old New Hampshire chicks were placed on the litter and on the wire. Thirty chicks were placed in each pen on the thirty-second day (eleven days after removal of the chicks that had been given *S. pullorum* orally). These chicks were kept on the litter and on the wire for forty-five days. No deaths that could be attributed to *S. pullorum* occurred in this time. In fact, the total mortality was less than 1 per cent.

TABLE 3—Plate Counts on SS Agar for the Persistence of Salmonella Pullorum in Litter (Trial 2)

Days after start of exper.	Number of organisms per gram of litter: New cob Pen 1	New cob Pen 2	Old B-U-L Pen 3	Old B-U-L plus lime Pen 4
2	38,000	30,000	22,000	20,000
8	184,000	130,000	16,800	20,000
12	850,000	730,000	13,000	8,000
15	92,000	80,000	0	0
17	20,000	30,000	0	0
21	25,250	36,000	0	0
23	25,000	47,000	0	0
25	34,000	28,000	0	0
33	10,000	23,000	0	0
39	16,500	12,000	0	0
47	10,500	6,000	0	0
59	2,500	4,100	0	0
67	1,200	800	0	0
70	0	0	0	0
76	0	0	0	0

Trial 2 was started near the end of April and was a duplicate of trial 1 in most respects. A second lot of 24-hour-old New Hampshire chicks was given 0.25 ml. of *S. pullorum* orally. The total mortality in this trial was 83.6 per cent (table 1). After twenty-one days, the chicks that survived were removed, the feed and water trays were sterilized, but the litter was not disturbed. In this trial, 24-hour-old chicks (30 per pen) were placed on the litter two hours after the surviving chicks had been removed. These chicks were observed for sixty-eight days and no deaths occurred that could be attributed to *S. pullorum*. The mortality in this trial was about 1 per cent.

It was not expected that deaths would occur on the old built-up litter since at the time the chickens were placed on the litter the organisms had disappeared (tables 2 and 3). Why the chicks that were placed on the new cob

litter did not contract pullorum disease is a matter of conjecture.

All the chicks were checked for serum antibodies by the rapid plate method but no positive results were obtained. In trial 1, attempts were made to determine if coproantibodies were present but negative results were also obtained.

Salmonella Gallinarum.—Salmonella gallinarum attacks not only the very young chicks but those of all ages. Thus, the chickens remaining from trial 1 were used for the *S. gallinarum* experiment. These chickens were 45 days old at the start of this work.

All chickens were removed from pens 1 and 3. The litter was sprayed in these two pens with a 24-hour broth culture of the organism. Each pen, which was 5 ft. by 7 ft., was sprayed with 1,500 ml. of the broth culture (8,600,000,000 organisms per milliliter). This particular strain was obtained from the Veterinary Laboratory, Department of Bacteriology, The Ohio State University. The litter was not disturbed and forty-eight hours later the chickens were replaced on it (30 per pen). This gave the following experimental conditions: pen 1, new cob litter, sprayed with *S. gallinarum;* pen 2, new cob litter—control; pen 3, old built-up litter, sprayed with *S. gallinarum;* pen 4, old built-up litter, control; pen 5, wire—chickens fed 0.25 ml. of *S. gallinarum;* control.

TABLE 4—Mortality of New Hampshire Chickens, 6 Weeks of Age, on Various Types of Litter Sprayed with Salmonella Gallinarum

	Total mortality (%)	*S. gallinarum* recovered at autopsy (%)
Pen 1, new cob litter sprayed with *S. gallinarum*	53	87
Pen 2, new cob litter control	10	0
Pen 3, old built-up litter sprayed with *S. gallinarum*	33*	40
Pen 4, old built-up litter control	7	0
Pen 5, wire chickens fed 0.25 ml. of *S. gallinarum* by mouth	33	100

*Includes 2 sick chickens, cause of illness not determined; 30 chickens in each pen.

Table 4 shows the results of this experiment when *S. gallinarum* was sprayed on the litter. It will be noted that there was a higher mortality among the chickens on new cob litter (53 per cent) than on old built-up litter (33 per cent). All chickens that died were autopsied and material from the liver and heart's blood was streaked on McConkey's agar plates to determine if *Salmonella gallinarum* was

TABLE 5—Persistence of Salmonella Gallinarum in Litter and Mortality by Days of New Hampshire Chickens

	Plate Count of Litter by Days																
	1 day	2	10	12	16	20	26	36	42	45	54	63	68 70
	6,000,000	4,000,000	3,500,000	1,000,000	2,000,000	40,000	16,000	20,000	8,300	2,100	2,600	800	0
Pen 1, new cob sprayed with *S. gallinarum*						1	1 1 1 1 2	1*	1 1 1 1 1	1*	1		
	0	0	0	0	0	0	0	0	0	0	0	0	0
Pen 2, new cob control											1*	1*	
	310,000	360,000	50,000	43,000	30,000	0	0	0	0	0	0	0	0
Pen 3, old built-up sprayed with *S. gallinarum*						1* 1		1 1	2*	1*			2 sick not dead
	0	0	0	0	0	0	0	0	0	0	0	0	0
Pen 4, old built-up control							1*		1*				
Pen 5, wire-chicks fed *S. gallinarum* orally			1 1 1		1	1	1 2 1						

Salmonella gallinarum not recovered at autopsy; in pen 5 the chickens were given 0.25 ml. orally (250,000 organisms); 30 chickens in each pen; chickens 6 weeks old at start of experiment.

present. This was accomplished by selecting typical colonies and then inoculating differential culture media.

Table 5 shows the plate count of *S. gallinarum* from the two types of litter and the death rate per day. It is of interest to observe again that the organisms disappeared much more rapidly from the old built-up litter (twenty days) as compared with the persistence of the organism in new litter for sixty-three days.

The agglutinating titers of the blood of those chickens remaining at the end of this experiment were determined. These chickens were 6 weeks old at the start of the work and 14 weeks old at its conclusion. The titer of the serum of those chickens on old built-up litter was higher (maximum of 1: 5120) than of those on new cob litter (maximum of 1: 320) or the controls that were fed the organisms (maximum of 1: 320). What significance this has, if any, has not been determined.

Discussion

Even though *S. pullorum* persists longer in new cob litter than in old built-up litter, the mortality is not increased. Also, it should be emphasized that the death rate does not increase when built-up litter is used. Whether or not it is a matter of dosage with both types of litter has not been established; however, the significant fact is that *S. pullorum* disappears more rapidly from old built-up litter than it does from new cob litter. This suggests that pullorum disease is difficult to transmit by means of the litter.

Salmonella gallinarum, when sprayed on the various types of litter, disappeared more rapidly from old built-up litter than from new cob litter. The death rate with 6-week-old chickens was also higher with the new litter than it was with the chickens kept on old built-up litter.

Whether or not other pathogenic bacteria will be reduced in numbers in built-up litter has not been investigated. On the basis of these experiments, built-up litter is superior to new cob litter in reducing the mortality rate of 6-week-old chickens when exposed to *S. gallinarum*.

A study of the factors responsible for the reduction in the number of pathogenic organisms and the reduction in mortality of chickens on built-up litter is underway.

Summary

1) *Salmonella pullorum* and *Salmonella gallinarum* disappear from old built-up litter more rapidly than they do from new cob litter.

2) *Salmonella pullorum* in the concentrations present in the litter in these experiments did not cause pullorum disease in New Hampshire chickens when exposed as day-old chicks.

3) When litter was sprayed with *S. gallinarum* and 6-week-old chicks introduced after forty-eight hours, the mortality rate was lower among the chicks on old built-up litter than in those on new cob litter.

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Some Effects of Oral Exposure of English Sparrows to Newcastle Disease Virus

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SINCE THE recognition of Newcastle disease (ND) *per se* in this country in 1944,[2] it has become one of the foremost problems of the poultry industry. Its appearance in relatively isolated poultry enterprises gave impetus to the opinion that free-flying birds were closely associated with the dissemination of the disease. This attitude resulted in decreased interest in sanitary efforts to confine ND to infected premises. The studies reported here were undertaken to help determine the role of free-flying birds in the spread of ND. The English sparrow (*Passer domesticus*) was chosen for study because of its presence in large numbers in close association with poultry flocks the year around over the entire United States, with the exception of a few areas of extremes in climate.[16] The virus of ND has been reported to be pathogenic for sparrows in India by Sahai,[18] who stated, "The disease is also pathogenic for sparrows, pigeons, and crows." Certain textbooks[15, 17] state that sparrows are susceptible. These statements are probably based on Sahai's assertion that sparrows are susceptible.

Numerous articles have appeared in the literature by workers in foreign countries reporting circumstantial evidence that ND occurs in free-flying birds.[1, 13, 19, 20] Attempts made on four occasions to isolate Newcastle disease virus (NDV) from crows in India[5] failed to reveal the presence of the virus, which represents one of the rare instances in which such attempts were made. Naturally occurring ND in free-flying birds in foreign countries has been reported in an adult gannet,[22] as evidenced by isolation of the virus. Reports of the natural disease in free-flying birds in the United States, based on isolation of the virus, have been made on a 12-day-old starling[6] and pheasant chicks,[12] the latter having been in captivity.

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MATERIALS

Sparrows were trapped on four locations near Lafayette, Ind. Each sparrow was tested for the presence of hemagglutination-inhibition (HI) antibodies. Only apparently healthy birds were used.

Two strains of NDV were used, namely the California 11914 strain and one recently isolated from the field and designated as the Sullivan (SVN) strain. In all instances, the virus employed was contained in the amnio-allantoic fluids of chicken embryos that had died from the infection. The SVN virus used was obtained from the second and third egg passages.

Plasma samples with and without antibodies for NDV were obtained from chickens. The antibodies were obtained in response to formolized 11914 virus.

Chicken red blood cells in 1.0 per cent suspension in phosphate-buffered (pH 7.8) sodium chloride solution (0.85%) were used in hemagglutination (HA) and HI tests.

METHODS

All sparrows were exposed to NDV by introducing 0.1 ml. of saline diluent containing NDV slowly into the oral cavity of each bird.

In instances in which blood was tested, as much as possible was withdrawn from the humeral veins and the heart. Sodium citrate was used as the anticoagulant. About 2 ml. of blood was the maximum amount that was removed from each sparrow at any one time. Blood was withdrawn from the heart by inserting through the thoracic inlet a 1-in., 20-gauge needle attached to a tuberculin syringe.

In tests for virus in sparrows that were destroyed or died, the brain, liver, and lungs of each bird were removed and extracts prepared for inoculation into embryonating chicken eggs. The individual tissues were ground and thoroughly mixed with 3 ml. of saline and stored at —28 C. The contents of the intestinal tract were also removed and mixed with 2 ml. of saline prior to storage at —28 C.

Extracts of brain, liver, lungs, feces, and intestinal contents were prepared uniformly for

inoculation of embryonating chicken eggs by the addition of penicillin[7] and streptomycin.[7,21] The final concentrations of the antibiotics were 2,500 units of penicillin and 25 mg. of streptomycin per milliliter. One-half-milliliter amounts of the antibiotic mixture were added to equal volumes of the extracts to be tested. These mixtures were incubated at room temperature for at least one hour before inoculation into eggs. The eggs were inoculated intraallantoically with 0.2 ml. of the materials. Fluids from all embryos which died between twenty-four hours and eight days following inoculation were tested for HA activity. Fluids of dead embryos showing HA activity were retested for HA activity in the presence of known NDV-negative serum and NDV antiserum. It was assumed, because of experience with the NDV strains used, that the embryo deaths occurring at times other than these were not due to NDV. The culture mediums employed for the detection of bacteria in egg materials were bacto-tryptose agar and thioglycollate and nutrient broths.

The procedure used in conducting the HA test followed, with slight modifications, the methods described by the Bureau of Animal Industry (BAI).[23] In this procedure, 0.25 ml. of a 1.0 per cent suspension of chicken red blood cells was mixed with 0.25 ml. of buffered saline and 0.25 ml. of dilutions of the extra-embryonic fluids.

A procedure used in the *alpha* HI test of sparrow blood was a modification of a test observed by Moses and Gustafson[14] to be applicable to chickens, wherein whole blood was used directly in the tube HI test. Two-tenths milliliter of sparrow blood was mixed with 4.8 ml. of buffered saline, and 0.5-ml. amounts of this mixture were combined with 0.25 ml. of virus. The *alpha* HI test used in all other instances was essentially that as suggested by the BAI.[23]

Infectivity-neutralization (IN) tests were conducted as recommended elsewhere;[23] four eggs were inoculated with each test material.

Recoverability of Newcastle Disease Virus from Tissues and Intestinal Contents—This study was planned to evaluate the invasibility of the virus for certain tissues and to determine whether or not it is present in intestinal contents. Two trials were run in series to evaluate these points. In the first trial involving 14 sparrows, each was exposed to $10^{3.5}$ lethal dose $(l.d.)_{50}$ of SVN virus. Two birds were killed by exsanguination every other day beginning on the third and ending on the eleventh day following exposure. In the second trial, 13 birds were exposed to $10^{7.5}$ $l.d._{50}$ of SVN virus, and 2 birds were killed each day beginning on the second and ending on the sixth day. In both trials, blood and extracts of the brains, livers, and lungs of the birds were tested for virus. In addition, in the second trial, intestinal contents were also tested. From birds that died following exposure, intestinal contents and tissues (with the exception of whole blood) were tested for NDV in the same fashion. Four birds which remained alive at nineteen days postexposure in the first trial were exsanguinated and the plasma was tested for HI antibodies.

RESULTS

Of 102 materials tested, NDV was recovered from 18. Thirteen of the virus recoveries were made from 90 materials tested from the 20 sparrows which were destroyed, and five virus recoveries were made from the 12 materials from 3 sparrows which died (7, 10, and 12 days postexposure) during the second trial. The virus was recovered from 7 of 12 birds which were killed two to five days following exposure and 2 which died on the seventh and tenth days, as shown in table 1. Recoveries were made from six livers, six lungs, three blood samples, two intestinal contents, and one brain.

Tests for HI antibodies in blood taken before exposure of the sparrows showed none to be present, and very low and questionably significant levels of HI antibodies were found in 3 of 4 birds killed on the nineteenth postexposure day. Of these 3 birds, 2 had developed two HI units and 1 had developed one unit.

The concentrations of NDV in the sparrow materials were indicated by recovery of the virus from the following dilutions: 10^{-3} of one brain and one intestinal contents; 10^{-1} of one liver and two lungs; and the initial preparation of five livers, four lungs, three blood samples, and one intestinal contents.

Recoverability of Newcastle Disease Virus

TABLE 1—Distribution of 18 Isolations of Virus from 9 Sparrows from Tests of 102 Specimens from 23 Birds

Trial (No.)*	Manner** and time of death days postexposure	Material yielding virus
1	K 3	Lungs, liver, and blood
	K 3	Lungs and liver
	K 5	Blood
2	K 2	Liver
	K 4	Lungs, liver, and blood
	K 5	Lungs
	K 5	Lungs and liver
	D 7	Intestinal contents
	D 10	Lungs, liver, intestinal contents, and brain

*These trials apply to the section on Recoverability of Newcastle Disease Virus from Tissues and Intestinal Contents. **K indicates that the bird was killed, and D shows that the bird died of possible effects of the virus.

from Feces—While this study primarily involved tests for virus in feces, tests were also made for virus in other materials and for antibodies to determine whether or not infection had occurred.

Three groups of 4 birds each were exposed *per os* to one of three virus materials containing $10^{7.0}$, $10^{4.0}$ or $10^{1.0}$ l.d.$_{50}$ of SVN and placed in cages according to the inoculum received. Four other birds that were not exposed were placed in a fourth cage as controls. Starting five days postexposure, but omitting the sixth day, and continuing through the fourteenth day, samples of feces were collected from the birds by pairs. For two hours on each of these days, the pairs of birds were placed on wire in 4,000-ml. beakers. The feces fell through the wire and into a glass container in the bottom of the beaker.

Tests for virus were conducted in chicken embryos, as previously indicated, and later, because of difficulties encountered, in chickens. These difficulties arose from the presence of an embryo-lethal factor in the fecal preparations, which were prepared by mixing 0.5 ml. of saline with each 200 mg. of feces. Attempts were made to determine the general nature of the factor by bacteriological studies, filtration, serial transfer in eggs, variation of the route of inoculation of embryos, and use of fresh rather than stored feces.

One-day-old chickens were given the feces *per os*. Each of 6 birds received 2.0 ml. of pooled fecal samples that were negative for virus in embryo tests or whose possible virus content was obscured by embryo deaths from other factors. On the thirty-fifth day postexposure, blood samples were taken from the chickens and tested for HI and IN antibodies. Previous studies with day-old chickens from the same source showed the development of ND following enteral exposure to approximately 1.0 l.d.$_{50}$ of SVN. Five days postexposure, the virus was isolated from their brains, livers, and spleens.

RESULTS

The inoculations of material from 40 of the 72 fecal samples and repetitions of 19 of the 40 samples into 236 eggs was followed by death of 135 (57%) within nineteen hours after inoculation and a total of 165 (69%) within forty-three hours. Tests of fluids from eggs for HA activity indicated virus to be present in the initial extract of one sample obtained from sparrows nine days after exposure to $10^{7.0}$ l.d.$_{50}$ of virus. This activity was inhibited by NDV antiserum. Bacteriological examination of the fluids of representative eggs indicated that the deaths of embryos with HA-negative fluids were due to factors other than contamination.

Attempts to determine the nature of the factor in feces that was lethal for embryos indicated that it was of small size and probably inanimate. The inoculation of 172 embryos with fecal extracts filtered through Swinny filters was followed by death of 88 (51%) within eighteen hours. No evidence of HA activity was demonstrated in egg fluids, and the results of bacteriological tests were comparable to those observed earlier. Evidence that any NDV present did not pass through the filters was shown by the failure of embryos to die following inoculation with filtrates made from materials containing as much as $10^{8.0}$ l.d.$_{50}$ of virus. Attempts to transfer the lethal factor serially in embryos failed. Likewise, inoculations of extracts of fresh fecal material onto chorioallantoic membranes of chicken embryos did not alter the pattern of embryo deaths due to this factor.

The chickens exposed to the pooled feces showed no observable symptoms of ND, nor did any HI or IN antibodies become demonstrable.

Tests for virus in brains, livers, lungs, and intestinal contents of 13 sparrows revealed none to be present. Two of the birds died nine and ten days after inoculation with either the largest or smallest amount of inoculum, and 2 of the 4 controls died on the sixth and fifteenth days of the experiment. The other 9 birds originated in the several inoculated groups, having been destroyed twenty-four to thirty days postinoculation.

Tests for IN antibodies in sparrows revealed titers that appeared to be correlated with the amount of virus to which they were exposed and/or to the interval between exposure to virus and collection of blood. Of 6 birds bled eighteen days postexposure, 3 exposed to $10^{7.0}$ l.d.$_{50}$ of virus had antibody titers of at least 100 or 1,000 units, and 3 exposed to $10^{1.0}$ l.d.$_{50}$ of virus had none, 10, or 100 units. Two sparrows exposed to $10^{4.0}$ l.d.$_{50}$ of virus and bled thirty days postexposure had antibody titers of at least 10,000 units. Tests conducted at this time on 2 control birds revealed 100 units in 1 and less than ten in the other.

Attempts to Transmit Newcastle Disease from Sparrows to Chickens by Simulated Natural Means.—Four sparrows were orally exposed to approximately $10^{5.5}$ l.d.$_{50}$ of SVN virus

in each of two trials, and likewise, 4 birds were exposed to $10^{5.75}$ l.d.$_{50}$ of 11914 virus in the first trial and to $10^{5.0}$ l.d.$_{50}$ of 11914 virus in the second trial. These sparrows were placed in cages on top of brooders containing 6 susceptible day-old chickens in such manner that the feces of the sparrows fell into the feed and water available to the chickens. In each trial, 4 sparrows which had not been exposed to NDV were placed in a similar manner over 6, day-old chickens as controls. As environmental controls, 12, day-old chickens were placed in a brooder in each of the two trials without the presence of sparrows or contact with NDV.

In both trials, blood samples were taken from each chicken and individually tested for HI and IN antibodies on the thirty-fifth day of the experiment. The brains, livers, and lungs of the sparrows which died during the trials were tested for virus. Sections of some tissues from these birds were prepared for histopathological study.

Results

There was no evidence of transmission of NDV from the sparrows to the chickens. Symptoms suggestive of ND were not observed in any of the chickens nor were any IN or HI antibodies found.

In the first trial, the 4 sparrows exposed to the 11914 virus and 1 of the uninoculated sparrows died. The former died fifteen, sixteen, seventeen, and twenty days postexposure, and the uninoculated bird died on the twenty-second day. Tests of the tissues for NDV showed the virus to be present in the lungs of 3 birds that had been exposed to the virus.

During the second trial, all 8 of the sparrows exposed to 11914 or SVN viruses and 1 of the uninoculated birds died. The sparrows exposed to 11914 virus died five, ten, eleven, and fourteen days after exposure. The birds exposed to SVN virus died one, three, four, and sixteen days postexposure. The uninoculated sparrow died on the third day. Tests for virus revealed it to be present in the liver and lungs of the sparrow that died on the fourth day after exposure to SVN and of the 1 that died on the fifth day after exposure to 11914 virus. The virus was not recovered from any of the other tissues.

Sections of 57 tissues from 11 sparrows of this trial were examined for microscopic lesions. Four of the birds had been inoculated with $10^{3.0}$ l.d.$_{50}$ 11914 virus and 4 with $10^{5.3}$ l.d.$_{50}$ SVN virus; 3 were controls. The most noteworthy observations were made from the sections of liver, spleen, kidneys, and lungs.

The liver of each of the 8 exposed birds showed pathological change. Diffuse leukocytic infiltration was present in three livers, increased size of lymphoid foci and diffuse lymphocytic infiltration in two, and increased size of lymphoid foci alone in one. Marked cirrhosis was present in the liver of 1 bird which died one day after inoculation. Evidence of early degenration of hepatic cells appeared to be present in three livers and, in two of these, passive congestion and focal central necrosis were also observed.

Of five spleens from exposed sparrows, two showed infiltration of immature lymph cells. In one, the infiltration was associated with enlargement of the spleen to at least twice normal size and, in another, with marked enlargement.

Kidneys from 4 of 5 exposed sparrows showed albuminous degeneration and desquamation of the epithelium of the collecting tubules.

Pneumonia characterized by cellular exudate in the aveoli and leukocytic infiltration of the perialveolar connective tissue was present in five of six lungs from exposed sparrows.

Discussion

The premise that English sparrows are susceptible to ND and may be involved in its dissemination tends to be supported by the work presented here. Susceptibility is evidenced in the data by virus recovery from various tissues, feces, and intestinal contents, deaths, development of antibodies, and micropathology. That sparrows may be involved in the spread of NDV is evidenced by the recovery of NDV from the feces of a pair of birds, in spite of difficulties encountered with a chicken embryolethal factor in the sparrow feces, and from the intestinal contents of several others.

The data indicate that the virus invaded and propagated in the tissues of the birds. The virus was recovered from the lungs, liver, brain, and blood at various times; in the case of lungs, up to twenty days postexposure. The recovery of NDV from the feces of 2 sparrows nine days postexposure and from intestinal contents of others up to ten days postexposure also supports this premise and gives evidence that the gastrointestinal tract is a portal of exit for NDV

and thereby provides a potential means of spread.

Evidence indicates that some of the birds died from the effects of NDV. Seventeen deaths occurred among 28 birds given the opportunity to live as long as twenty-three days. Micropathological lesions found in exposed birds that died were not observed in control birds that died or were destroyed.

Antibody formation was observed in experimentally exposed birds, but antibodies were not found in any prior to such exposure. Infectivity-neutralization antibodies were demonstrated in the birds which survived exposure to intermediate or large amounts of NDV. Only low amounts of HI antibodies were demonstrated in the 4 birds tested nineteen days after exposure to large amounts of NDV. Tests were made only for HI antibodies immediately following collection of sparrows, as the size of the sparrow presents a limiting factor in the procurement of experimental materials from individuals. The inability to demonstrate HI antibodies may have been due to a characteristically low capacity to produce such antibodies, as suggested by the low titers demonstrated following exposure to medium and large amounts of virus.

The tests simulating natural exposure of susceptible day-old chickens to exposed sparrows did not demonstrate transmissibility of ND, even though the virus was recovered from some of the sparrows which apparently died from the effects of NDV. There is no clear explanation for this result.

The feeding of chickens with pooled feces of exposed sparrows did not result in transmission of the disease. This may have been due to the repeated freezing and thawing of the feces, necessitated by their use in other experiments.

The age of the sparrows was not considered in the studies. Drawing inference from the variable susceptibility of chickens, turkeys, and mice[10] at various age levels, it is possible that such differences may also be obtained in sparrows.

The degree of natural resistance of sparrows to NDV, revealed by these studies, suggests that the sparrow is at present of minor importance in the epizoötiology of ND. However, it seems possible that serial passage of the virus through sparrows and/or entry of the virus by means other than *per os* may gradually or abruptly adapt strains of NDV into higher virulence for sparrows and make sparrow-to-chicken transmission common.

Since these studies were completed, a report of naturally occurring ND based on isolation of the virus has been made on a Great Horned Owl of hitherto unknown susceptibility.[8] Also, experimental evidence has been obtained on the ability of orally exposed *Passer melanurus*[9] and house sparrows and Java sparrows[11] to eliminate the virus for variable periods.

SUMMARY

Studies were made to assess the effects of oral exposure of Newcastle disease virus (NDV) on the English sparrow (*Passer domesticus*).

1) The virus was recovered from the liver, lungs, blood, brain, or intestinal contents of 14 of 58 exposed sparrows.

2) Assessment for NDV in fecal samples of exposed sparrows by inoculation of chicken embryos was hindered by an embryolethal factor; however, the virus was recovered by this method from one of 72 samples.

3) Demonstration of transmission of Newcastle disease from sparrows to chickens was not accomplished by close association of the birds, although evidence of infection of the sparrows was shown.

4) Histopathological studies revealed lesions in certain tissues which appeared to be associated with infection by the virus. The results are interpreted to signify that the sparrow has a measure of natural susceptibility to NDV introduced *per os*.

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Chemotherapy of Enterohepatitis of Turkeys
III. Comparative Value of 2-Amino-5-Nitrothiazol and 2-Acetylaminonitrothiazol in Prevention and Treatment

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THE VALUE OF 2-amino-5-nitrothiazol and 2-acetylaminonitrothiazol in the prevention of enterohepatitis of turkeys has been reported by various investigators.[1-4] Jungherr and Winn[3] reported that 2-amino-5-nitrothiazol in the ration at a concentration of 0.1 per cent was an effective treatment in clinically affected birds. McGregor[4] found that a concentration of 0.05 per cent of the drug in the ration was not an effective treatment but did have prophylactic value. Grumbles, Boney and Turk (unpublished data) found 2-amino-5-nitrothiazol given continuously in an all-mash ration at the concentration of 0.05, 0.04 and 0.03 per cent to be effective in preventing mortality caused by enterohepatitis. This study showed that the drug had little value in treating enterohepatitis when medication was started after an outbreak of the disease.

The object of this study was to further evaluate the prophylactic and therapeutic value of 2-amino-5-nitrothiazol and to compare its activity with 2-acetylaminonitrothiazol.

MATERIALS AND METHODS

The holding facilities consisted of a shed-roof type brooder house with a dirt floor. The house was 8 ft. by 180 ft. and was divided by wire partitions into 15 pens. One 500-watt electrical brooder was used in each pen.

Broad Breasted Bronze poults were started and maintained in battery brooders until placed on test. The birds in the first experiment were 4 weeks old and those in the second experiment were 2 weeks old when started on experiment.

The birds for each pen were selected by random sampling and identified by wing bands. The number of birds in a pen in the first experiment varied from 43 to 50. Fifty birds were used in each pen in the second experiment with the exception of one control group which contained 65 birds. The first experiment originally contained 15 pens; however, during the third week of the test a wind storm completely demolished the brooder house. The building for pens 1 through 8 was rebuilt and the pens reconstituted within three days. The facilities could not be rebuilt in time to continue the experiment in pens 9 through 15. Because of this, only the prophylactic phase could be completed.

In a previous experiment, litter from a house of chicken hens known to harbor *Heterakis gallinae* produced a high, uniform mortality from blackhead when used as litter in turkey pens. In this study, the ground which served as a floor for the house had been used as a range for chickens for several years. In addition to this, a uniform amount of litter from the above mentioned henhouse was placed in each pen.

In the prophylactic study, medication was started at the time of exposure and given continuously in all-mash ration for the duration of the experiment. In the therapeutic study, the drugs were administered in each pen after mortality from blackhead had occurred and was given continuously in an all-mash ration for fourteen days.

Experiment 1 contained pens 1 through 8. The birds in pens 1, 3, and 5 received 2-amino-5-nitrothiazol at the concentrations of 0.05, 0.04 and 0.03 per cent, respectively. Pens 2, 4, 6 and 8 received 2-acetylaminonitrothiazol at the concentrations of 0.05, 0.04, 0.03 and 0.02 per cent, respectively. Pen 7 was the non-medicated control.

Experiment 2 contained 15 pens of birds. Pens 1A through 8A was a repetition of experiment 1 and the respective groups received the same level of medication as in experiment 1. The birds in pens 9A through 15A were given the drugs on a therapeutic basis and medication was not started until enterohepatitis had occurred in the pen. The birds in pens 9A and 11A were given 2-amino-5-nitrothiazol at the concentrations of 0.2 and 0.1 per cent, respectively. Pens 10A, 12A and 13A received 2-acetylaminonitrothiazol at the

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concentrations of 0.2, 0.1 and 0.05 per cent, respectively. Pens 7A, 14A and 15A were the nonmedicated controls.

Because of economic factors and the availability of a market for turkey broilers, the experiments were discontinued when the birds were 10 weeks old. Experiment 1 covered a period of six weeks while experiment two covered a period of eight weeks.

The birds were weighed at the beginning of the experiment and at weekly intervals. Weekly feed consumption was recorded for each pen. Birds that died during the experiment were autopsied and an attempt made to determine the cause of death. The diagnosis of enterohepatitis when made was based on gross lesions.

RESULTS

The results of this study are summarized in table 1. It is apparent that 2-amino-5-nitrothiazol and 2-acetylaminonitrothiazol were effective in preventing mortality from enterohepatitis when the drugs were started at the time of exposure to *Histomonas meleagridis* and given continuously in an all-mash ration. Only one death from enterohepatitis occurred in experiment 1; this was in the pen which received 0.03 per cent of 2-acetylaminonitrothiazol. There was a 22 per cent mortality in the nonmedicated control pen.

In the prophylactic study of experiment 2, 1 bird from the group on 0.04 and 1 from the group receiving 0.02 per cent of 2-acetylaminonitrothiazol died from enterohepatitis. In the three nonmedicated controls pens there was an enterohepatitis mortality of 6, 6, and 20 per cent or an average of 11.5 per cent.

In the therapeutic part of experiment 2, the enterohepatitis mortality in the birds receiving 2-amino-5-nitrothiazol at a concentration of 0.2 and 0.1 per cent was 8 and 8 per cent, respectively. The mortality in the birds receiving 2-acetylaminonitrothiazol at the concentrations of 0.2, 0.1 and 0.05 per cent was 8, 8, and 4 per cent, respectively. The enterohepatitis mortality in the three nonmedicated control groups was 6, 6, and 20 per cent or an average mortality of 11.5 per cent.

Neither of the two compounds at the concentrations used significantly affected body weights, feed consumption or feed utilization; therefore these data are not included.

SUMMARY

Data from two experiments comprising 1,132 turkey poults are presented on the value of 2-amino-5-nitrothiazol and 2-

TABLE 1—Prophylactic and Therapeutic Use of 2-Amino-5-Nitrothiazol and 2-Acetylaminonitrothiazol Against Enterohepatitis of Turkeys

Experiment 1—Phophylactic								
Pen No.	1	2	3	4	5	6	7	8
No. birds	46	47	46	44	46	50	45	43
Medication*	0.05%–*1	0.05%–*2	0.04%–*1	0.04%–*2	0.03%–*1	0.03%–*2	None	0.02%–*2
Enterohepatitis mortality (%)	0	0	0	0	0	2	22	0
Experiment 2—Phophylactic								
Pen. No.	1A	2A	3A	4A	5A	6A	7A	8A
No. birds	50	50	50	50	50	50	50	50
Medication*	0.05%–*1	0.05%–*2	0.04%–*1	0.04%–*1	0.03%–*1	0.03%–*2	None	0.02%–*2
Enterohepatitis mortality (%)	0	0	0	2	0	0	6	2
Experiment 2—Therapetuic								
Pen No.	9A	10A	11A	12A	13A	14A	15A	
No. birds	50	50	50	50	50	50	65	
Medication*	0.2%–*1	0.2%–*2	0.1%–*1	0.1%–*2	0.05%–*2	None	None	
Enterohepatitis mortality (%)	8	8	6	8	4	6	20	

* *1: 2-amino-5-nitrothiazol; *2: 2-acetylaminonitrothiazol.

acetylaminonitrothiazol in the prevention and treatment of enterohepatitis in turkeys.

These data indicate that both drugs are effective in preventing mortality from enterohepatitis when started at the time of *Histomonas meleagridis* exposure and given continuously in the ration. Under the conditions of this study, 2-amino-5-nitrothiazol and 2-acetylaminonitrothiazol were of about equal prophylactic value.

Under the conditions of this experiment, neither of the two drugs were of significant value in reducing enterohepatitis mortality when medication was delayed until an outbreak of the disease had occurred.

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Electron Micrographs of Newcastle Disease Virus Propagated in the Cotton Rat (Sigmodon Hispidus)

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ELECTRON MICROSCOPE studies were conducted to determine the morphology of the Newcastle disease virus (NDV) after intracerebral passage in the cotton rat *(Sigmodon hispidus)*. Reagan and Brueckner[1] showed a predominance of tailed virus forms in purified egg-adapted NDV, Colorado strain (BAI I 1946). These tailed forms were also found by Reagan and coworkers[2] in bat-adapted NDV (California strain 11,914) after one passage in 10-day embryonating chicken eggs.

EXPERIMENTAL

Six-hundredths milliliter of allantoic fluid from 10-day embryonating White Leghorn eggs infected with the 16th embryo passage of NDV, Colorado strain (BAI I 1946) was inoculated into the right frontal lobe of each of 6 cotton rats (*Sigmodon hispidus*) 21 days of age. Forty-eight to seventy-two hours after inoculation the cotton rats showed typical Newcastle disease symptoms: irritability and malaise followed by rhythmical contraction of the muscles of the limbs and spreading over the entire body, and paralysis of the hind legs. When nervous symptoms developed in a rat, the brain was removed aseptically, ground with alundum in a small mortar, and diluted to a 10 per cent suspension with physiological saline. This virus-bearing suspension was injected intracerebrally into each of 6 cotton rats as described above. Typical Newcastle disease symptoms appeared in the rats of the second passage from two to seven days after intracerebral inoculation. The infected rats were killed, and the same procedure was carried out in making a 10 per cent brain suspension. This virus-bearing material from each of the rat passages was inoculated into the allantoic sac of five 10-day embryonating chicken eggs. All embryos died within seventy-two hours. A pool of the allanto-amniotic fluid from the dead embryonating eggs infected with each rat passage was prepared. The presence of virus in each of these two pools was demonstrated, and the virus was verified to be that of Newcastle disease by a neutralization test using embryonating chicken eggs as the test host. Newcastle disease-immune chicken serum completely neutralized the virus; normal chicken serum had no effect.

Each pool of allanto-amniotic fluid was centrifuged in a horizontal centrifuge for five minutes at 2,000 revolutions per minute. The sediment was discarded and the supernatant fluid was recentrifuged for three hours at 50,740 revolutions per minute in a Spinco ultracentrifuge, Model E. There was a 2 degree change in the temperature of the refrigerated outer jacket. The sediment was resuspended in 0.5 ml. of 3 per cent saline for ten minutes, placed on parlodion prepared screens, shadowed with chromium[3] at arc tan 3/12 and examined under an RCA EMU electron microscope.

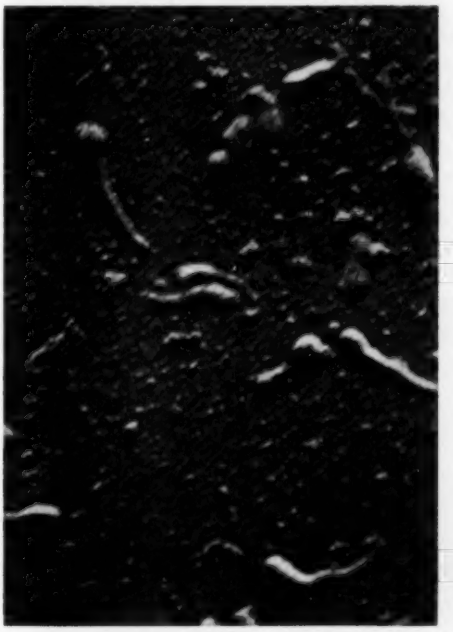

Fig. 1—Newcastle disease virus from the brain of an infected cotton rat, (Sigmodon hispidus), after one subculture in embryonating eggs. x 80,000.

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RESULTS

Various fields chosen for electron microscope examination showed many virus particles with tail structure and some without tail structure. Figure 1 shows NDV from the first cotton rat passage after cultivation in embryonating eggs. The electron micrographs (fig. 2) of NDV from the second cotton rat passage after cultivation in embryonating eggs show the virus particles to be similar to those in figure 1.

Fig. 2—Newcastle disease virus from the brain of an infected cotton rat, (Sigmodon hispidus), after one subculture in embryonating eggs. x 95,000.

SUMMARY

Newcastle disease virus, Colorado strain (BAI I 1946) was carried for two serial passages in cotton rats inoculated by the intracerebral route. Allanto-amniotic fluid from embryonating chicken eggs infected with rat brain suspensions from both passages was used in electron microscopic studies. The morphology of the virus did not appear to be altered by passage through cotton rats. The virus was verified to be that of Newcastle disease by neutralization tests in embryonating chicken eggs.

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Morphological Observations by Electron Microscopy of Pseudorabies Virus (Aujeszky Strain) After Propagation in the Rabbit

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THE AUJESZKY STRAIN of pseudorabies virus used for this study was furnished by the American Type Culture Collection of Washington, D. C. This virus in infected rabbit brain had originally been obtained from A. Aujeszky in 1931. Susceptible hosts (cattle, cats, dogs, and ordinary laboratory animals) die following inoculation by any route. Swine develop a mild, nonfatal illness when infected subcutaneously or intramuscularly, and the serums of such animals, following recovery, is viricidal. This Aujeszky strain from Hungary is identical with the pseudorabies (mad itch) virus causing disease in the United States.[1] Before receipt at this laboratory, the virus had been passed several times in rabbits. The exact number of passages in rabbits is unknown.

When received at this laboratory, the glycerinated rabbit brain was ground in a mortar with alundum and diluted to a 10 per cent suspension with normal physiological saline. Two albino rabbits (weight 3 lb.) were inoculated intracerebrally with 0.1 cc. of the 10 per cent virus-bearing brain suspension. These rabbits showed nervous symptoms and excessive salivation in twenty to thirty hours. When symptoms appeared, the rabbits were destroyed and the brains were removed aseptically. Three other rabbits (weight 3 lb.) were injected intracerebrally with a 10 per cent suspension of normal rabbit brain. No symptoms were seen in these normal controls. Two of the normal rabbits were destroyed thirty hours after inoculation. The brains were removed aseptically as mentioned previously. The third control rabbit appeared normal after a ten-day observation period and was discarded. The pools of infected rabbit brains and of normal rabbit brains were ground with alundum and diluted to 10 per cent suspensions with physiological saline. The two suspensions were then subjected to five-minute centrifugation in an angle centrifuge at 2,000 revolutions per minute. The supernatants were filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation for three hours at 194,000 gravitational force under refrigeration. The temperature of the refrigerated outer jacket stayed constant at —8 C. during the centrifugation. After the three-hour centrifugation period, the supernatants from the specimens were discarded, and the sediment from each specimen was resuspended in 1.0

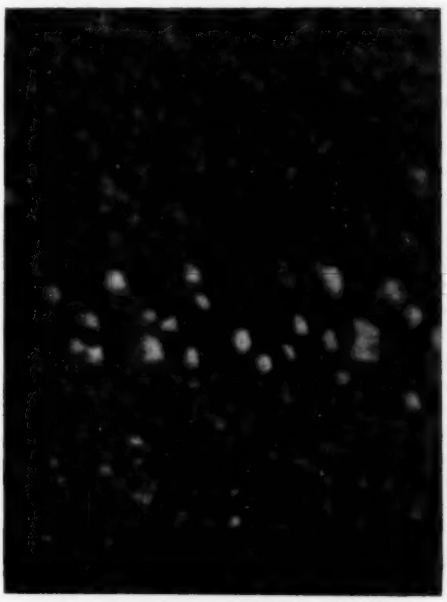

Fig. 1—Pseudorabies virus shadowed with chromium at arc tangent 2/10. x 40,000.

From the Live Stock Sanitary Service Laboratory, Maryland State Board of Agriculture, University of Maryland, College Park.

Associate professor of veterinary virology (Reagan), instructor (Schenck), bacteriologist (Harmon), and director (Brueckner).

cc. of physiological saline. Several drops of each specimen were placed on several parlodion film supports which had been prepared forty-eight hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium[2] at

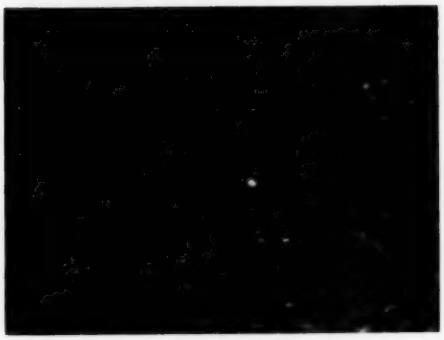

Fig. 2—Normal rabbit brain material at arc tangent 2/10. x 40,000.

arc tangent 2/10 and examined under the R.C.A. electron microscope, type E.M.U. The balance of the material of each suspension was injected intracerebrally into 2 rabbits (weight 3 lb.).

The rabbits injected intracerebrally with the concentrated suspension of normal rabbit brain appeared normal and were discarded after a seven-day observation period.

The rabbits injected intracerebrally with the concentrated infected material showed nervous symptoms and became prostrate after forty-four hours. These rabbits were destroyed; the brains were removed aseptically, pooled, ground, and diluted to a 10 per cent suspension. This suspension was injected intradermally into 3 normal rabbits (weight 3 lb.). These rabbits showed the following symptoms after forty-eight hours: scratching and biting of the skin at the site of inoculation, due to the pruritus produced by the virus. Salivation and grinding of teeth followed. Death occurred in all 3 rabbits within seventy-two hours. These rabbit brains were removed aseptically, pooled, ground, and diluted to a 10 per cent suspension. This suspension was injected intradermally into 3 unvaccinated guinea pigs and into 3 guinea pigs previously immunized intramuscularly with the subculture of pseudorabies virus which had been obtained from the American Type Culture Collection. After three days, the unvaccinated guinea pigs showed symptoms of central nervous system involvement characteristically found in this disease, while the immunized group showed no nervous symptoms. This confirmed the concentrated virus to be pseudorabies virus.

Electron microscopic examination of the concentrated suspension of the brains of the normal rabbits revealed no virus-like particles. These controls were screened carefully. Upon examination of the concentrated suspension from the infected brains, uniform virus-like particles were demonstrated, as shown in fig. 1. These bodies measured 90 to 100 μ by direct measurement and appeared spherical with somewhat irregular contour. Figure 2 is a micrograph of a normal rabbit brain in which no virus-like particles were seen.

The virus of pseudorabies, as measured by ultrafiltration, was 120 μ in diameter.[3]

SUMMARY

Studies by electron microscopy of a concentrated virus-bearing brain suspension of rabbits infected with pseudorabies (Aujeszky strain) showed the virus to be somewhat spherical with a diameter between 90 and 100 μ. These bodies could not be demonstrated in concentrated normal rabbit brain subjected to the same procedure of concentration and examination. This concentrated infected material, upon being injected into normal rabbits intracerebrally, produced typical symptoms of pseudorabies. Virus suspensions from the brains of these rabbits injected intradermally into other rabbits produced pruritus and death within seventy-two hours. The virus in the concentrated material was infectious for unvaccinated guinea pigs but had no effect on guinea pigs previously immunized against pseudorabies virus.

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Continuous Ink-Writing Kymograph Unit for Teaching and Research in Veterinary Physiology and Pharmacology

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TEACHING and research in the field of veterinary physiology and pharmacology is facilitated by continuous recording equipment. Recordings of the changes in body functions should indicate not only the dramatic effects but should also indicate the latent period and the time necessary for return to normal function.

In many instances, where smoked paper recordings are made, only the dramatic effects can be recorded. For example, if one is to study the effect of hemorrhage on blood pressure and the return of blood pressure to normal as a result of intravenous infusion, a long recording period is necessary. Drugs with a delayed action period or drugs that modify function for a long time can not be studied adequately unless continuous recordings are made.

In order to overcome the disadvantages inherent in the use of smoked paper kymograph equipment, we have constructed a continuous ink-writing kymograph unit, using relatively inexpensive standard equipment. This equipment has been in use in our laboratories for the past three years and has proved itself to be superior to smoked paper equipment for most of our experiments.

The essential features of the equipment can be noted by examination of fig. 1 and

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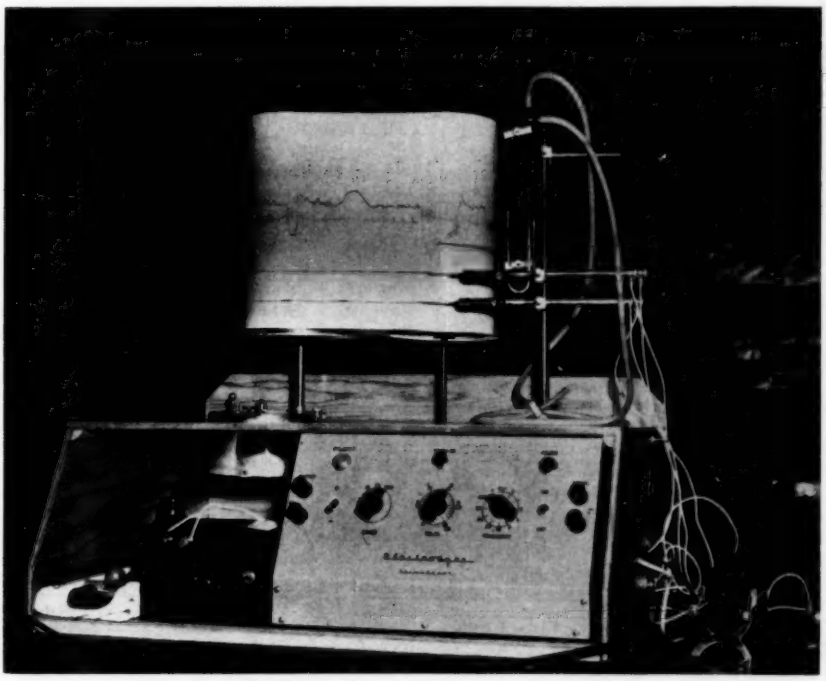

Fig. 1—Essential features of the ink-writing kymograph unit.

2. The standard Harvard kymograph pulls paper from a roll (11-in. paper). In order for the kymograph to pull a large roll of paper, the friction plate of the kymograph must be fastened to the sleeve by means of a metal pin or by fusion of these parts. Eighteen inches of the tracing can be observed during the progress of the experiment. Following the experiment, the entire tracing can be studied. Experiments lasting several hours can be conducted without the addition of a new supply of paper. The continuous movement of the paper keeps the pens running free. Placing the kymograph in a well in the cabinet insures proper alignment of the kymograph with respect to the paper supply. The cabinet is constructed in such a manner as to accommodate the time clock mechanism, the electrodyne stimulator, batteries and wire connections, and a space for various attachments and instruments. This arrangement facilitates keeping the equipment clean and reduces equipment damage that often re-

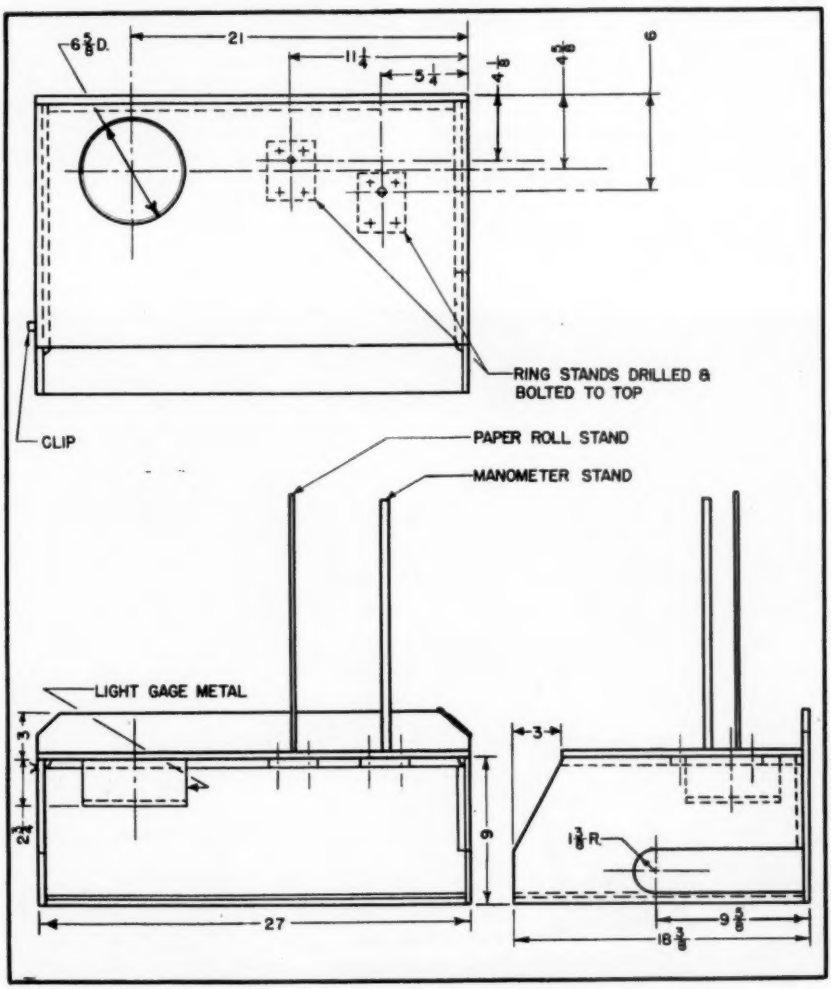

Fig. 2—Mechanical details of the ink-writing kymograph unit.

sults when equipment is openly exposed on the laboratory desks. This compact unit also allows more free desk space for other equipment. The inductorium is shown outside the cabinet in fig. 1. This instrument can be placed inside the cabinet on the instrument tray if desired.

The compact unit for student laboratories has proved itself to be efficient, has given the student a greater appreciation of time intervals involved in drug action, and has encouraged him to think in terms of rate of change of body functions rather than in terms of the dramatic effects alone.

Studies of Pseudorabies Virus (Aujeszky Strain) in the Rhesus Monkey (Macaca Rhesus)

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Two milliliter of this virus-bearing brain material was inoculated into each of 2 Rhesus monkeys (weight 5 lb.) by one of the following methods: intracerebrally, intradermally, and rectally. The response of the monkeys inoculated with this virus is recorded in table 1.

The monkeys injected intracerebrally with the infected material showed nervous symptoms and became prostate after five days. The monkeys exposed rectally and intradermally showed slight nervous symptoms and some scratching on the seventh day. The monkeys from all groups were killed on the seventh day; the brains were removed aseptically and stored at —40 C. Three days later the brains of all monkeys were thawed, ground separately, and diluted to 20 per cent suspensions with physiological saline. Five-tenths milliliter of each suspension was injected intradermally into individual rabbits. The 2 rabbits inoculated with the brain suspensions from the intracerebral series developed typical symptoms of pseudorabies on the third day. The other 4 rabbits remained normal during a ten-day observation period and were discarded.

Neurtalization tests using guinea pigs as the test host confirmed the virus in the brain suspensions from the monkeys of the intracerebral series to be pseudorabies virus.

DISCUSSION

From the results of the preceding experiment it was evident that the pseudo-

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rabies virus produced only a mild infection in monkeys inoculated intradermally and rectally. However, when injected intracerebrally this virus proved to be highly pathogenic. Hurst[3] had previously found this virus to be pathogenic for monkeys by intracerebral, intracisternal, and intrasciatic injections but not by intradermal, intramuscular, and intravenous inoculations.

TABLE I—The Response of Monkeys to Pseudorabies Virus by Different Routes of Inoculation

Monkey	Route of inoculation	Incubation period	Symptoms
1	Intracerebral	5	Paralysis
2	Intracerebral	5	Paralysis
3	Intradermal	7	Scratching and nervous symptoms
4	Intradermal	7	Scratching and nervous symptoms
5	Rectal	7	Scratching and nervous symptoms
6	Rectal	7	Scratching and nervous symptoms

SUMMARY

The Rhesus monkey (*Macaca rhesus*) was susceptible to the Aujeszky strain of pseudorabies virus by intracerebral exposure. The virus in monkey brain suspensions from the intracerebral series was confirmed to be pseudorabies virus by neutralization tests using the guinea pig as the test host. Monkeys infected intradermally and rectally with this pseudorabies virus showed slight nervous symptoms and some scratching. However, rabbits inoculated with brain suspensions from these animals showed no evidence of infection.

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